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T cells, Dendritic cells and the human immune response to viruses and vaccines

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Thesis submitted for examination of Doctor of Philosophy (PhD) September 2005

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Abstract

The work presented in this thesis investigates human immune responses to measles virus (MV). Identification of potential MV T cell epitopes for use as part of a new generation component vaccine could overcome problems associated with live vaccines in early life. HLA-A2*0201-restricted epitopes, both predicted sequences and previously identified epitopes, were used to stimulate peripheral blood samples from immune adult donors. Responses were investigated using the IFNγ ELISpot. This approach failed to identify dominant epitopes. Therefore methods of enhancing presentation were investigated. To screen for immunodominant epitopes, overlapping 15mer peptides from 3 major MV proteins F, H and NP were used, looking for responses to peptide pools and individual peptides. Although some responses were seen this did not identify any dominant epitopes.

In vivo boosting of the response was undertaken by vaccinating HLA-A2 individuals with MMR vaccine. PBMC and serum samples were stored from several time points' pre and post vaccination. Cell phenotyping, serology and response to peptides, along with in vitro expansion of antigen specific cells, were used to assess response in vaccinees. In vitro expansion of cells responding to MV proteins was to be achieved using adenoviral vectors containing cDNA coding for individual MV proteins. This might allow expression of antigen in autologous dendritic cells (DC) via the natural route of infection without encountering the cytopathic effect of live MV on DCs. This work, although unsuccessful, led to the observation that adenovirus-infected DCs suppress T cell proliferation, leading to a novel body of work looking at the effects of adenoviral infection on human DCs.

In parallel with this work, similar methodology was used to investigate T cell responses to an OMV based meningococcus B vaccine in vaccinated donors. However it transpired that this vaccine did not stimulate an immune response in the majority of donors investigated.

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Abbreviations

βgal	β- galactosidase protein
A	Adenosine
Ab	Antibody
APC	Antigen presenting cell
BCIP/NBT	5-bromo-4-chloro-3-indolyphosphate and nitroblue tetrazolium
BCR	B cell receptor
BSA	Bovine serum albumin
C	Cytosine
CAR	Coxsackievirus and adenovirus receptor
CFSE	Carboxyfluorescein Diacetate Succinimidyl Ester
Ci	Curies (disintigrations/minute)
CM	Complete medium (RPMI 10% FCS)
Cpm	Counts per minute
CMV	Cytomegalovirus
CTL	Cytotoxic T Lymphocyte
DC	Dendritic cell
DMSO	Dimethyl sulphoxide
dNTP	deoxy- nucleotide-triphosphate
dsRNA	double stranded ribo-nucleic acid
EBV	Epstein Barr virus
ELISA	Enzyme linked immunosorbant assay
ELISpot	Enzyme linked immunospot assay
EPI	Expanded programme on immunization
F	Fusion protein (MV)
FACS	Fluorescence activated cell sorting
FcR	Fc Receptor
FCS	Foetal calf serum
FDG	Flourescein di(B-D-glucuronide
FITC	Fluorescein Isothiocyanate
FM	Freezing medium
G	Guanine

g	Gravity
GM-CSF	Granulocye Macrophage colony stimulating factor
H	Haemagglutinin protein (MV)
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPA	Health Protection Agency
HRP	Horse radish peroxidase
ICH	Institute of Child Health
IDO	indoleamine 2, 3-dioxygenase
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
ITAM	Immunoreceptor tyrosine based activation motif
ITSM	Immunoreceptor tyrosne based switch motif
LAL	Limulus Amebocyte Lysate assay
LPS	Lipopolysaccharide
LSTHM	London School of Hygeine and Tropical Medicine
M	Matrix protein (MV)
MenB/C	Meningitidis B/C
MHC	Major histo-compatibility complex
MMB	Mini Macs buffer
MMR	Measles Mumps Rubella vaccine
MOI	multiplicity of infection
MV	Measles virus
NCPV	National collection of pathogenic viruses
NK	Natural killer cell
NP	Nucleoprotein (MV)
OMV	Outer membrane vesicle
P/S/glu	Penicillin/ streptomycin/ glutamine
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline/ Tween

PCR	Polymerase Chain Reaction
PE	R-Phycoerythrin
Pfu	Plaque forming unit
PHA	Phytohaemagglutinin
PI	Propidium Iodide
PMA	Phorbol 12-Myristate 13-actetate
PRN	Plaque reduction neutralisation assay
PVDF	polyvinylidine diflouride
QR	Quantum Red
Rad	Recombinant adenovirus
RSV	Respiratory syncytial virus
SBA	Serum bactericidal assay
SCID	Severe combined immuno-deficiency
SD	Standard deviation
SSPE	Sub acute sclerosing pan encephalitis
T	Thymidine
TCID	Tissue culture infectious dose
TBE	Tris/borate/EDTA
TCR	T cell receptor
TEMED	NNNN tetramethyl ethylene diamine
TGFβ	Tumour growth factor beta
Th	T helper cell
TLR	Toll like receptor
TNFα	Tumour necrosis factor alpha
Tr1	T regulatory cell type 1
Treg	T regulatory cell
TT	Tetanus toxoid protein
UCL	University College London
X-gal	5-Bromo-4-Chloro-3-indolyl β-D-Galactopyronoside
XLA	X-linked agammaglobulinaemia

Chapter 1 Introduction

1.1 The Immune System

Everyday, humans come into contact with a huge range of potentially infectious organisms. However illness only occurs relatively rarely. The human immune system has evolved side by side with these microorganisms and has developed a wide variety of both general and highly specific mechanisms to protect humans from infection. The first line of this defence are the obvious physical barriers preventing microorganism entry to the body, i.e. the epithelia. Internal epithelial surfaces such as those of the gastrointestinal and respiratory tracts also secrete mucus which contains glycoproteins known as mucins which can prevent microorganisms from binding to the epithelial surface and therefore prevent invasion or colonisation. These mucosal surfaces are further protected by the movement of epithelial cilia which expels any microorganism caught in the flow of mucus.

There are other chemical barriers such as lysozyme in tears and saliva and antimicrobial molecules such as defensins which reside in the respiratory tract and in the small intestine (Krisanaprakornkit et al., 2000). Another factor that protects humans from colonization with pathogenic microorganisms is the presence of the natural flora of non pathogenic bacteria which often have a survival advantage and are thought to compete with the pathogenic microorganisms for nutrients and attachment sites on epithelial cells. These commensals can also themselves secrete anti microbial molecules which prevent the colonization by other species. It is when this barrier is breached by either damage to the skin or exposure to a

particularly successful human pathogen, that the immune system is required to clear the infection (Ouellette, 1999).

1.1.1 Innate Immunity

Once the outer defences have been breached the next line of defence against infection is the innate arm of the immune system. This is a comparatively non organism specific recognition of invading pathogens which allow activation of specific pathways which lead to the destruction of the pathogen or subsequent activation of other branches of the immune system. This arm of the immune system although effective, does not become more effective upon subsequent re infection with the same pathogen, meaning that there is no "memory" of each infection.

1.1.1.1 Cell mediated innate immunity

There are several major cell types and molecular pathways involved in the innate protection of the human body to infection. One of the most important cells involved in this process are the phagocytes which are cells that can recognize and ingest whole microorganisms. Macrophages are the predominant phagocytic cell in the tissues. These macrophages are constantly reconstituted from monocytes migrating from the peripheral blood. All phagocytic cells recognize cell surface markers that can distinguish host cells from pathogenic cells. They do this via the expression of the mannose receptor, scavenger receptors and CD14 which is one of the receptors for lipopolysaccharide (LPS), a major immunogenic constituent of bacterial cell membranes (see section 1.1.2.1). Once bound to the phagocyte the active process of phagocytosis occurs where by the pathogen is enveloped in the

phagocytic membrane and sealed within a phagosome. This becomes an acidic environment and is fused with lysosomes, granules within the cell that contain antimicrobial products, to make a phagolysosome where the destruction of the pathogen occurs. Phagocytes can also release several other toxic products such as hydrogen peroxide and nitric oxide which are toxic to the bacterium (Aderem and Underhill, 1999).

Neutrophils are the other major phagocytic cell type. These are not normally resident in resting tissue, although when macrophages and other pro-inflammatory pathways are activated, chemotactic agents are released which recruit neutrophils and other cells into the area. This process, known as inflammation, leads to blood vessels becoming more permeable allowing delivery of cells and effector molecules to the area of infection. Neutrophils are short lived since the toxic nature of the reagents they use to kill pathogens can also damage the cell (Segal, 2005). Macrophages are longer lived, and survive, which enables them to process and present antigens derived from the phagocytosed pathogen to other cells which have been recruited to the area such as T cells which form part of the adaptive immune system (which is discussed later in section 1.1.2).

The main difference between a pathogenic and non pathogenic organism is the ability of that organism to overcome innate immune defences. This may be through sheer numbers, or more commonly many microorganisms have developed wide ranging strategies to avoid being immediately identified by macrophages. These strategies include bacteria coating themselves with a polysaccharide capsule, which is not recognized by receptors on phagocytes. Some intracellular bacteria

such as mycobacteria have developed the ability to prevent fusion of the phagosome with the lysosome and therefore can survive within macrophages (Ferrari et al., 1999).

Other organisms, particularly viruses, have the ability to infect different cell types and in order to avoid detection alter the cell in which they are replicating to either suppress immune responses as with measles (MV) as will be discussed later, or to down regulate cell surface expression of the Major Histocompatibility Complex I (MHC I) which would normally display intracellular protein products to T cells. This is discussed in detail in section 1.1.2.3. There is another cellular component of the innate immune system which combats this specific phenomenon. Rather than relying on the recognition of non self in order to combat pathogens, Natural Killer (NK) cells rely on recognition of self MHC I molecules in order for them not to kill cells, this allows them to remove altered cells from the body. This not only aids in the fight against viruses and intracellular pathogens that alter cells, but can also play a role in the detection and removal of other abnormal cells such as cancerous cells (Moretta et al., 2002). Once activated, NK cells also play a role at inflammatory sites by releasing cytokines and other inflammatory mediators.

1.1.1.2 Complement

There is another major system in place that can recognize and destroy invading pathogens. This is the complement system, which is made up of a large number of plasma proteins that react with each other in a cascade mechanism, which both amplifies the response and can lead to the death of the organism. The proteins reside all over the body in a pro-enzyme form. Each enzyme in the pathway is the

substrate of the enzyme before it in the cascade. When cleaved the enzyme becomes active and then cleaves and activates the next enzyme in the pathway. The smaller cleavage products in the cascade also act as pro inflammatory mediators and can recruit phagocytes to the site of infection.

There are three main pathways through which the complement system can be activated. These are the classical pathway, the mannose binding lectin (MBL) pathway and the alternative pathway. All of these pathways can result in the effector functions of the complement system which are: recruitment of phagocytes and mediating inflammation, formation of a membrane attack complex, which directly causes the lysis of certain pathogens or results in the opsonization of the pathogen by phagocytes (Tomlinson, 1993). A schematic diagram of the activation steps in the complement pathway and the different proteins involved is shown in figure 1.1.

At the centre of all of the pathways is the C3 protein. It is the cleavage of C3 that releases C3a, which is a peptide mediator of inflammation, but more importantly activates C3b, which can either bind directly to the target cell surface where it is the ligand for C3b receptors on some phagocytic cells marking the cell for phagocytosis. Either the pathogen is phagocytosed or C3b binds C3 convertase which forms C5 convertase which cleaves C5 to make C5a, the most important inflammatory mediator and C5b which initiates the late events in complement activation which leads to the formation of the membrane attack complex. The membrane attack complex which involves proteins C6, C7, C8 and C9, forms a

pore in the target cell surface which can result in the death of that organism (Bhakdi and Tranum-Jensen, 1991;Tomlinson, 1993).

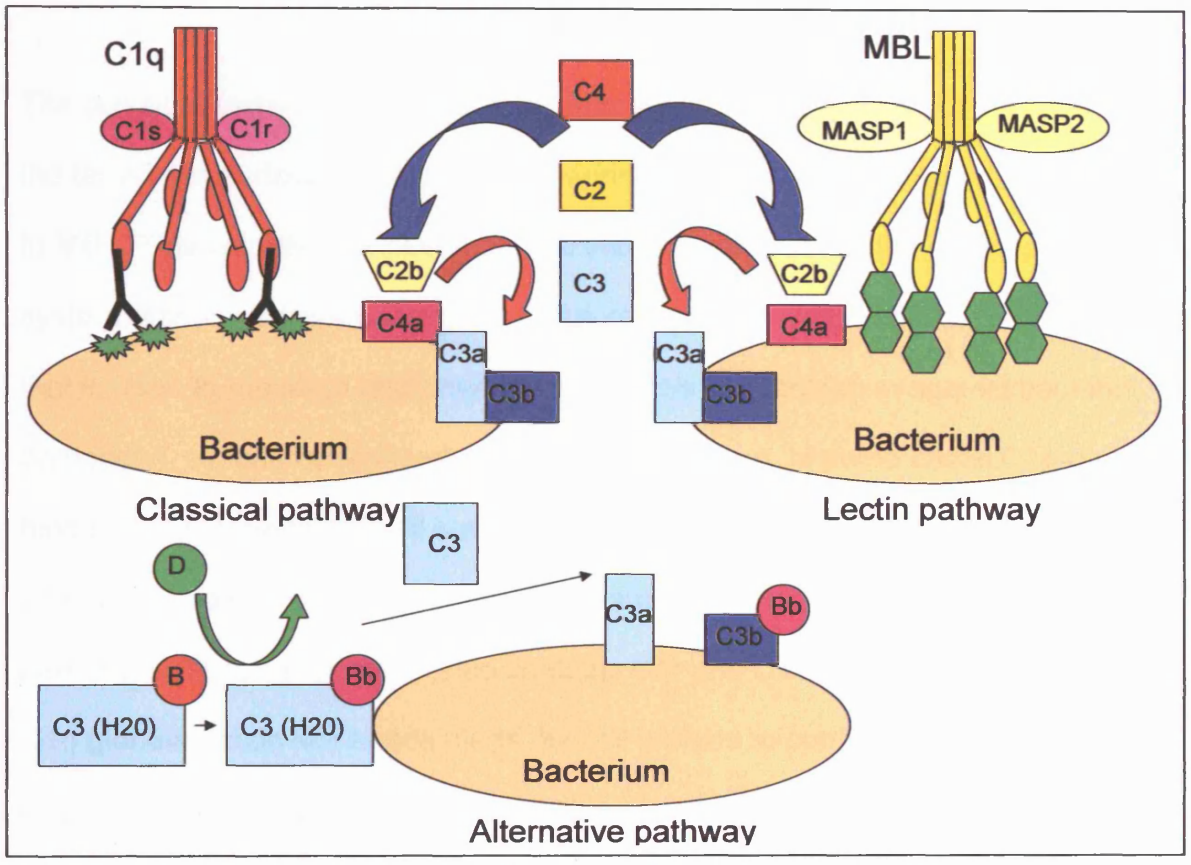


Figure 1.1: A schematic diagram of the complement system and the three pathways through which it is activated. Adapted from (Fujita, 2002).

Thus, activation of the complement pathway may occur through three major pathways. The alternative and MBL pathways are the two "innate" pathways. In the MBL pathway a collectin protein mannose binding lectin (MBL) binds mannose residues and certain other carbohydrates on the surface of pathogens which results in the recruitment of MBL-associated serine proteases MASP-1 and MASP-2, these then cleave C2 and C4 which result in the hydrolysis of C3 to C3a and C3b (Vasta et al., 1999).The alternative pathway is activated via spontaneous hydrolysis of C3; this occurs spontaneously in plasma however C3b can not

activate the alternative pathway unless it can combine with proteins B and D. This can only occur once C3b undergoes a conformational change caused by being bound to the surface of a pathogen (Platonov, 1999;Tomlinson, 1993).

The classical pathway requires the binding of antibodies specific for molecules on the target cell surface which are then bound by C1q, another collectin that is similar to MBL. This links the adaptive humoral immune response with the complement system, and this reaction forms the basis of the serum bactericidal assay (SBA), that is used to measure responses of individuals to vaccination against bacterial pathogens, which is discussed further in section 7.1.4. In some cases C1q can also bind directly to the target cell surface and can activate the classical pathway of complement directly, without the need for antibodies for certain pathogens. C1q is part of the C1 complex which also contains C1r and C1s. If more than one of the C1q globular lectin like heads binds the cell surface, a conformational change occurs which activates C1r which in turn activates C1s. C1s can then act on C2 and C4 which results in the hydrolysis of C3 which activates the rest of the complement pathway (Cooper, 1985;Tomlinson, 1993).

These innate mechanisms used to combat infection rely on general recognition of pathogen structures as "non self" in order to facilitate killing. Successful pathogenic microorganisms have evolved mechanisms to evade recognition by the innate immune system and establish an infection. In order to successfully deal with such infection the immune system requires a more specific arm. This is known as adaptive immunity.

1.1.2 Adaptive immunity

Adaptive immunity consists of two main arms: cell mediated immunity, which involves destruction of infected cells by cytotoxic cells, and humoral immunity which involves the production of antibodies against pathogen components. Adaptive immunity is not an immediate early line of defence since it requires the expansion of antigen specific cells and their maturation into effector cells. However once this has occurred and the cells have expanded and cleared the infection, many of them die but some persist as memory cells, allowing the response to subsequent infection with the same pathogen to be quicker and larger (secondary response) than on first encounter (primary response). The major cell types involved are the T and B lymphocytes respectively. There is some cross over between these two lymphocyte subsets, as T cells are required to establish humoral immunological memory by B cells.

1.1.2.1 Antigen presenting cells (APC)

Antigen presenting cells (APC) are required to process and present antigens to T lymphocytes as T cells do no recognize cell surface antigens alone. T cells recognize short peptide fragments of proteins derived from pathogens that have been processed by the APC and presented in the context of a major histocompatibility complex protein (MHC) on the APC surface. This process is further discussed in section 1.1.2.3. When antigen is recognized by T cells on the surface of the APC, in order for an immune response to be initiated the APC must be in an activated state to provide co-stimulation for the T cells (Banchereau and Steinman, 1998).

Dendritic cells (DCs) are professional antigen presenting cells and are widely credited with playing a central role in the immune system (Banchereau and Steinman, 1998). DCs link innate recognition of general "danger" signals, the presence of pathogen associated molecules such as double stranded ribonucleotides (dsRNA) and lipopolysaccharide (LPS), with the adaptive immune response (Gordon, 2002;Schulz et al., 2000).This is achieved by the presence of pattern recognition receptors both on the surface and within intracellular organelles of the DC. Pattern recognition receptors include the Toll like receptors (TLR). This family of proteins was initially identified in the fruit fly drosophila, and named Toll receptors (Barton and Medzhitov, 2002).

Over 9 different human TLR have been identified. The ligands for some, but not all of these TLR molecules have been discovered, along with their different sites of expression within a cell and cell subtypes. For example TLR4, which recognizes LPS, is expressed on monocytes and myeloid DCs and not on plasmacytoid DCs, where as TLR7 and TLR9, which are involved in the recognition of viruses, are expressed on the surface of plasmacytoid DCs but not on myeloid DCs (Reis e Sousa, 2004a;Reis e Sousa, 2004b).This shows a functional segregation of receptors as plasmacytoid DCs are high type 1 interferon producing cells which is the key cytokine involved in the immune response to viruses (Saint-Vis et al., 1998).

DCs are distributed throughout most tissues and mucosal surfaces. They are constantly sampling the environment by internalizing molecules from the surrounding area. This occurs through several processes including

macropinocytosis. In this process, large volumes of surrounding fluid are taken up non –specifically along with receptor mediated phagocytosis of antigen by membrane bound molecules such as CD205 (DEC-205) and DC-SIGN (CD209) (Engering et al., 2002). DC-SIGN is also involved in the interaction between DCs and T cells by binding to ICAM-3 on the T cell surface (Geijtenbeek et al., 2000b). Interestingly DC-SIGN has been identified as a receptor for HIV entry into DCs (Geijtenbeek et al., 2000a). DCs display the processed antigen on their surface for recognition by T cells. However the DCs will only mature and migrate to lymph nodes if they have been activated by the recognition of pathogen derived molecules. DCs can then go on to secrete specific cytokines tailored to the type of immune response required to fight that pathogen. For example if TLR4 is ligated by LPS, this signals the presence of bacteria and so requires the production of T cell help in order for B cells to produce antibody. In contrast if TLR9 is ligated this may indicate the presence of a virus which would require a DC to produce type 1 interferons, leading to increased expression of MHC class 1 on the cell surface and promoting production of cytotoxic T cells (CTL) which can kill virus infected cells (Saint-Vis et al., 1998). It is in this way that the innate and adaptive arms of the immune system are tightly linked, with the production of a specific signal by the DCs determining the fate of T cells (Barton and Medzhitov, 2002).

The T cells activated by contact with DCs will be those that have recognised antigen on the surface of the DC via their specific T cell receptor (TCR) and also receive co-stimulation from the up regulated co stimulatory molecules on the mature DC surface. This combined with the production of specific cytokines tailored to the type of immune response required will stimulate previously naïve T

cells to develop into the necessary phenotype to fight the invading pathogen. It is thought that without co-stimulation on the surface of the APC, T cells encountering antigen are not activated and therefore DCs also play a role in the maintenance of self tolerance in the periphery either by the induction of anergy in the T cells (Mahnke et al., 2002), or through the production of specific cytokines that induce the differentiation of a different population of T cells known as T regulatory cells (Treg) which themselves can modulate immune responses (Munn et al., 2002). This is further discussed in section 1.1.2.3.

As DCs are central to the immune response, several pathogens including measles virus (MV) (section 1.3) are able to modulate the action of DCs to their own advantage increasing the ability of the pathogen to successfully infect the host (Bhardwaj, 1997;Xu et al., 2001). This is mainly through the suppression of responses by the decreased expression of the Th1 inducing cytokine IL-12 by DCs during an MV infection (Atabani et al., 2001;Marie et al., 2001). Many other viruses also interfere with the functions of DCs including RSV (Bartz et al., 2003), HSV (Pollara et al., 2003) and Adenovirus (Jonuleit et al., 2000). The latter will be discussed further in chapter 6. Another mechanism that viruses may use to manipulate the immune system is to induce the production of T regulatory cells to effectively suppress the immune response that would otherwise clear the infection; this will also be discussed further in chapter 6.

1.1.2.2 B lymphocytes

B lymphocytes or B cells, (so called because they develop in the bone marrow then mature in the periphery), can also act as antigen presenting cells. However there is

a much reduced capacity for B cells to prime T cells and direct the nature of an immune response compared to DCs. Once B cells recognize antigen in its natural conformation via the B cell receptor (BCR) they can internalize this antigen via receptor mediated endocytosis and process this antigen and present it to T cells in the context of MHC molecules. This allows for the recruitment of antigen specific T cell help to the B cell. In the case of a primary response this can occur on the surface of a DC which provides cytokines and appropriate co stimulation to drive the appropriate immune response. However during recall memory responses the B cell can present antigen to and activate, the specific T cell. This in turn can produce cytokines and co stimulation to provide the second signal to help differentiation and antibody production by the B cell (Parker, 1993).This is the basis for the difference between so called T cell independent and T cell dependent antigens. T cell dependent antigens require the T helper cell to provide the second signal for activation. T cell independent antigens bind the BCR and the second signal is provided by the antigen itself by recognition of a microbial cell constituent such as LPS (O'Rourke et al., 1997).

Although antibodies can be produced by B cells without T cell help which do provide some level of protection, (e.g. responses to polysaccharide antigens on the surface of capsulated bacterium), in order for the B cell to undergo proliferation and affinity maturation of their Ag specific receptor (surface Ig), T cell help is required (Parker, 1993). It is this phenomenon that can be manipulated using vaccination of polysaccharides conjugated to proteins to enhance memory and antibody affinity in the young (MacDonald et al., 1998). The initial phase of B cell activation usually occurs when a specific B cell is trapped in the T cell zone of the secondary

lymphoid tissue. This is a mechanism which ensures that the rare B and T helper cells which are specific for the same organism are more likely to come into contact. The primary phase of the B cell response involves proliferation of both T helper cells and B cells in what is known as a primary focus. After several days a proportion of these B cells will terminally differentiate into antibody secreting plasma cells, which migrate to the red pulp of the spleen. This provides a first wave of antibody responses that can disseminate into the periphery to help fight the infection. These plasma cells have a range of life spans: some may only persist for the duration of the infection, while others are very long lived and may account for the persistence of long term antibody responses (Manz et al., 1997).

Other B cells and T helper cells migrate to a primary lymphoid follicle where they form a germinal centre. This is where through successive rounds of somatic hyper mutation of Ig-coding sequences, and selection by antigen in germinal centres of the lymph nodes, affinity maturation of the B cells occurs (Berek et al., 1991). This is a very "resource expensive" process for the cell where at least 1 in 2 B cells will undergo apoptosis due to loss of affinity for antigen or through competition with higher affinity clones (Ziegner et al., 1994). The process results in a few clones with much increased affinity for the antigen. With help derived from Th2 cells, B cells undergo isotype class switching to enable them to make different immunoglobulin (Ig) subclasses (IgG, A, E). This involves the combining of the variable gene with different constant region coding sequences, coded for downstream which results in the progeny of one B cell having the ability to produce antibodies with the same specificity for antigen but of different isotypes. These are further described later in this section. These cells then go on to

terminally differentiate into Ig producing plasma cells or remain as B cell memory (Manz et al., 1997). This occurs both during the first encounter of the antigen as the primary response progresses and again during subsequent recall responses. This process is heavily reliant on the interaction between the cell surface proteins CD40 and CD40L. The process of signalling via CD40 to induce class switching is also dependent on cytokine production by T helper cells. This allows different pathogens to induce different proportions of the different isotypes. For example worms and parasites induce the IgE isotype possibly due to the production of cytokines such as IL-4 by Th2 cells.

On re-stimulation with antigen some of these memory B cells divide and differentiate quickly into plasma cells, while others return to the lymph nodes and undergo more rounds of affinity maturation (McHeyzer-Williams and Ahmed, 1999). Thus, unlike T cell memory, B cell memory increases in affinity on re-exposure to antigen. The main differences between memory and naïve B cells are the speed at which specific antibody can be produced, as well as an increase in affinity for the antigen and the Ig class of antibody produced. This changes from IgM at the start of the primary response to IgG and IgA later in the primary response and thereafter in the memory response (Ward and Ghetie, 1995).

The BCR is the membrane bound form of the antibody that the B cell will go on to secrete once it differentiates into a plasma cell. Antibodies are made up of four polypeptide chains, two heavy chains and two light chains. The light chains consist of two immunoglobulin protein domains, one variable and one constant. The heavy chains consist of three constant and one variable domain, two domains either side

of a flexible linker region. The variable domains arrange at the antigen binding region of the molecule to provide maximum antigen binding diversity as different heavy and light chain combinations can come together to give a variety of antigen specificity (Edelman, 1991). The variable domain is most variable in three regions which are the exposed loops between the immunoglobulin structural regions of the domain. These regions are specifically targeted for gene recombination and rearrangement combined with somatic hyper mutation that further diversifies the host repertoire of antibody (Fanning et al., 1996).

In germinal centres the gene rearrangements of the C regions of the heavy chain that result in an isotype switch occur. The first isotype of antibody produced by plasma cells is IgM. This is a low affinity antibody as B cells producing it have not undergone somatic hypermutation and affinity maturation. However since IgM molecules exist as pentamers, the overall avidity of the complex for antigen is high. For this reason IgM is found mainly in the blood, as pentamers are too large to diffuse into tissues. IgM is a potent activator of complement, so plays an important role in controlling blood borne infections. IgG, IgA and IgE are smaller in size and can therefore diffuse into the tissues they will have undergone affinity maturation and therefore act as monomers (IgA can exist as a dimer). IgG is the principal antibody isotype in the blood and can be further split into subtypes IgG1-4. These subtypes relate to the nature of the response and pathogen and whether the response is Th1 or Th2 driven (Clark, 1997). IgG is a potent activator of complement, opsonization and sensitization for NK killing of cells. IgA is mainly present in secretions at mucosal surfaces, and as there are no complement proteins or phagocytes in this environment, the main function of IgA is to neutralize

invading pathogens and block binding to mucosal surfaces therefore preventing infection. IgG can also act as a neutralising antibody (Ward and Ghetie, 1995). IgE is present at low levels and is bound avidly by mast cells which reside beneath the skin and mucosa. Triggering of mast cell activation results in release of potent mediators such as histamine which results in coughing and sneezing aimed at expelling infectious agents. However it is this part of the immune system that can become over sensitized to the common environmental antigens and plays a large role in allergy.

1.1.2.3 T lymphocytes

T cell precursors arise in the bone marrow. These migrate to the thymus and undergo most of their development and selection there, hence the name T lymphocytes. The development of T cells involves both positive and negative selection to ensure that the rearranged T cell receptor (TCR) binds to self MHC (positive selection) but that the TCR is not reactive against self derived peptides (negative selection) (Gascoigne et al., 2001). It is for this reason that T cells in a specific organism are 'MHC restricted', meaning that they will only recognize peptides in the context of self MHC molecules. During development, thymocytes progress from a double negative (CD4-CD8-) to a double positive stage, ie when they express both the co receptors CD4 and CD8. If the TCR is reactive to MHC class I, it looses the expression of CD4 and if it binds MHC class II, it looses the expression of CD8. Thus mature cells in the periphery express either CD4 or CD8 but not both (von Boehmer, 1988).

1.1.2.3.1 The T cell receptor (TCR)

The structure of the TCR and its interaction with MHC is central to the biology and function of T cells. The T cell receptor itself is made up of an α:β heterodimer structured homologous to an antibody molecule. Each chain consists of two domains, one constant and one variable. The membrane-distal ends of the molecule are exposed in order to recognize antigen. However this heterodimer alone cannot activate the T cell. The α:β heterodimer is part of the TCR complex which also contains two ε chains, one δ chain and one γ chain which makes up the CD3 molecular complex. CD3 plays a role of stabilizing the TCR at the cell surface and in signalling (Davis et al, 1988; Chothia et al, 1988).

1.1.2.3.2 Signalling of the TCR complex

Also assocated with the α:β heterodimer is a ζ chain homodimer which is responsible for most of the signalling into the T cell. The ζ chain homodimer has an intracellular domain that has six immunoreceptor tyrosine-based activation motifs (ITAMs). On TCR activation, due to antigen receptor binding the ITAM becomes tyrosine- phosphorylated and transmits the activation signal into the cell. Other chains within the CD3 complex also have ITAMs and can play a roll in signal transduction. Also associated with the CD3 complex is either CD4 or CD8 which act as co-receptors as they recognize and bind to either MHC II or MHC I respectively. The cluster of MHC molecules and co-receptors binding to the TCR and its associated molecules is known as an immunological synapse (Sidney et al., 2001). The TCR complex is shown in figure 1.2; the interaction is shown in figure 1.3a).

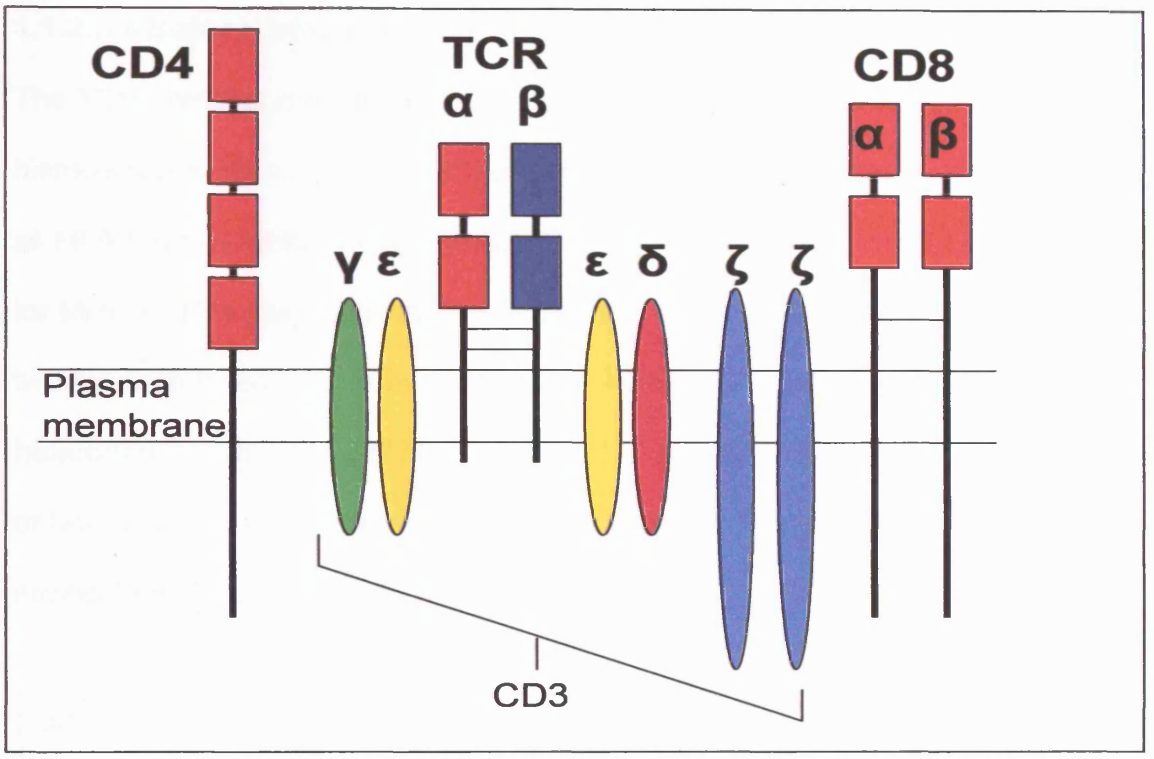


Figure 1.2: Schematic diagram of the T cell receptor complex showing the different chains and the signalling ITAMs and the CD4 and CD8 co receptors only one of which is expressed on either T helper or cytotoxic T cells, respectively.

1.1.2.3.3 Activation of the T cell- co-stimulation

Binding of the TCR complex to the MHC peptide complex is not sufficient to activate the T cell; a second signal resulting from the ligation of co-stimulatory molecules such as CD28 and CD80 is also required. This prevents activation of T cells to foreign peptides that are not associated with danger such as food derived antigens. Innate mechanisms which activate and up regulate co-stimulatory molecules as discussed in section 1.1.2.1 are responsible for activating the APC to give the second signal required for appropriate T cell activation.

1.1.2.3.4 Major Histocompatibility Complex (MHC)

The TCR complex only recognizes antigen in the context of a major histocompatibility complex (MHC) molecule. In humans the MHC alleles are known as HLA (human leukocyte antigen) A, B and C for MHC I and HLA-DR, DP and DQ for MHC II. This gives a possible combination of six MHC I and six MHC II alleles, two for each locus, one from each parent. MHC molecules are composed of a heterodimer. In the case of MHC I (figure 1.3) this is the combination of an α chain or heavy chain, with three domains, combined with the stabilising non variable molecule β_2 microglobulin.

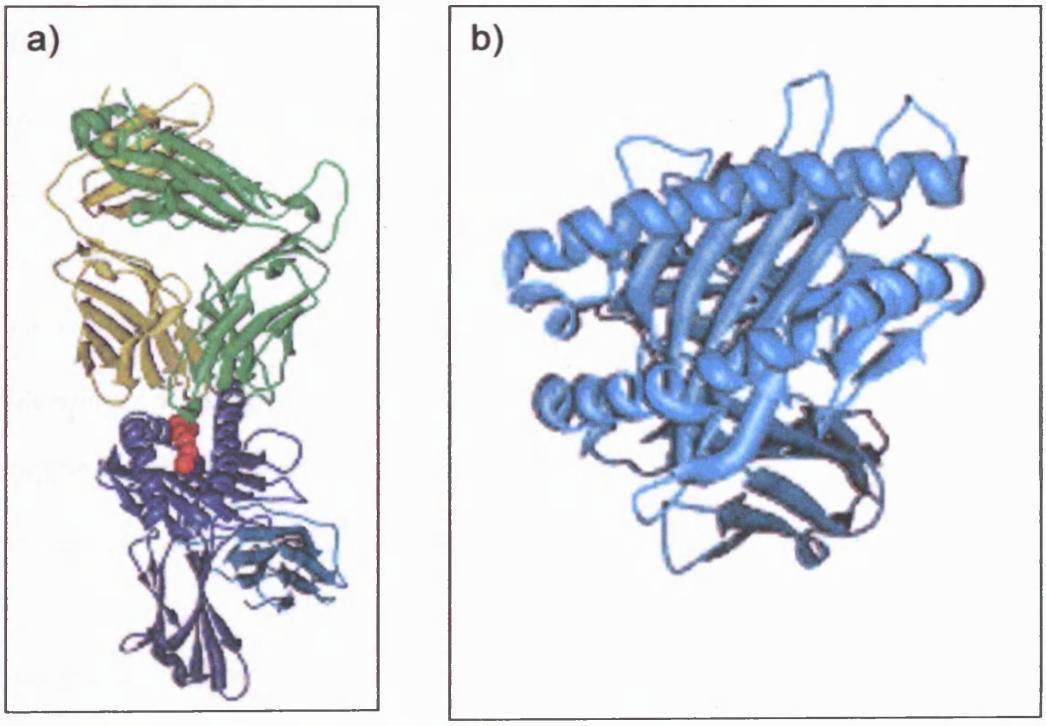


*Figure 1.3: Ribbon diagrams of a) HLA-A2*0201- Heavy chain in purple, β2 microglobulin in blue and peptide in red being recognized by an antigen specific TCR in yellow and green and b) the MHC class I binding groove as seen from above. Created in Chimera and reproduced with the kind permission of Barry Flutter.*

The α1 and α2 domains form a cleft which is known as the peptide binding groove (Bjorkman et al., 1987). The groove is sealed at both ends allowing only peptides of specific lengths (8-12 amino acids) to bind and be presented to T cells. The peptide groove is formed at its base by a β pleated sheet of protein secondary structure while the sides of the groove are made up of two α helices one from the α1 an one from the α2 domain of the heavy chain of the MHC I molecule. Binding of a peptide as part of the trimeric complex is required for the MHC molecule to be stable at the cell surface (Elliott et al., 1991;Townsend et al., 1990). This is shown in figure 1.3 for MHC I.

MHC II is also a heterodimer of an α and β chain. However each has two domains and the α1 and β1 domains combine to form the peptide binding groove. This results in a more open conformation and MHC II molecules can bind peptides of between 13-17 amino acids long. The individual binding grooves associated with each allele expose different amino acid side chains within the groove which while allowing a wide range of peptides from many organisms to bind, will only bind peptides with a specific motif that will fit in the binding groove. The important residues in each motif are known as anchor residues (Sidney et al., 2001).

1.1.2.3.5 T cell functions

The functions of cells expressing CD4 and therefore recognizing MHC II molecules, and CD8 therefore recognizing MHC I molecules, are very different. CD4 T cells are known as T helper (Th) cells which provide co-stimulation and cytokines to drive the differentiation of B cells and activation of other cells in the immune system. This will be further discussed later in this section. CD8 T cells are

known as cytotoxic T lymphocytes (CTL) and can directly kill virus infected or abnormal cells. For this reason the antigens that each type of T cell recognizes are derived from different sources. In health, only phagocytic cells such as macrophages, B cells and dendritic cells express MHC II. This is because peptides presented in the context of MHC II are derived from extra cellular antigen which enters the cell via endosomes and is degraded into peptide fragments before being loaded on to MHC II molecules in the endoplasmic reticulum and transported to the cell surface for recognition by CD4 T cells, this is shown in figure 1.4 (Pieters, 2000).

MHC I is expressed on the surface of most nucleated cells in the body. Expression of intra cellular proteins as peptides on class I MHC on the cell surface acts as a monitoring system for what is occurring inside the cell. The proteosome degrades intra cellular proteins which are then loaded on to MHC I molecules for presentation at the cell surface. This occurs with the cooperation of a variety of peptide loading proteins which are beyond the scope of this thesis (Flutter B, 2004). Therefore if the cell is infected with an intracellular pathogen the peptide presented derived from that pathogen will be recognized as foreign by a CTL and the infected cell will be destroyed halting the spread of the infection. This process is also shown in figure 1.4.

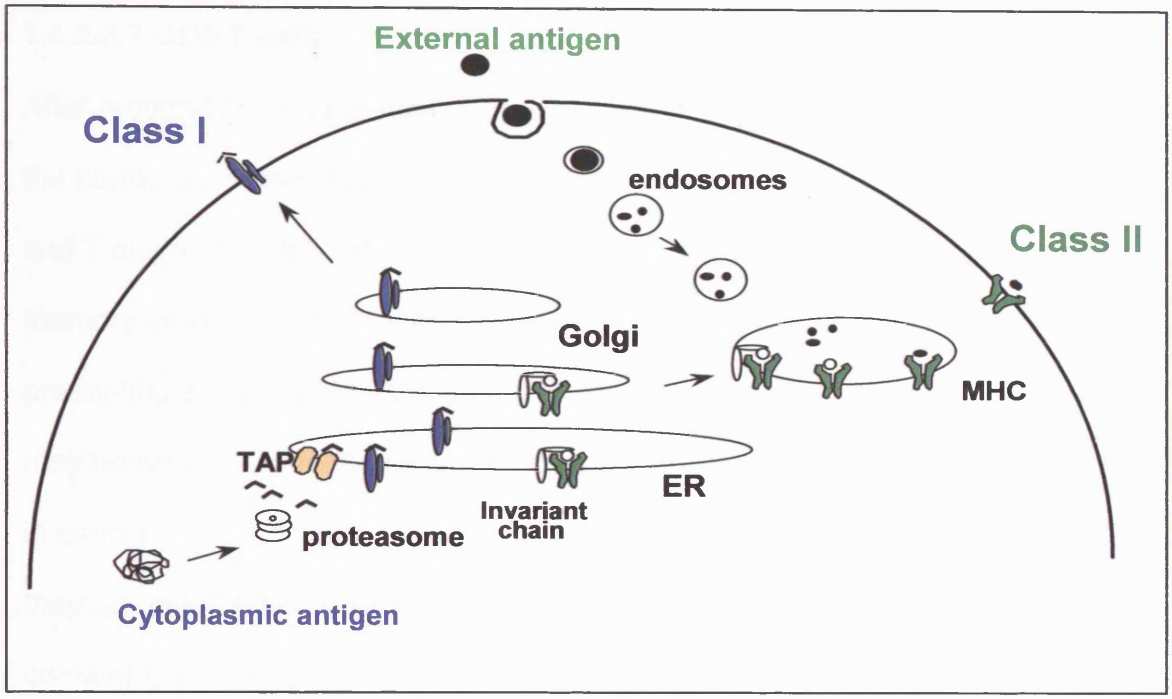


Figure 1.4: Diagram to show the different pathways of antigen presentation by MHC molecules. In blue is antigen from the cytoplasm digested in proteosomes and resulting peptides loaded onto MHC I molecules. In green is external antigen being engulfed and digested in endosomes and resulting peptides loaded onto MHC II molecules and presented at the cell surface.

1.1.2.3.6 Cross presentation

This system is complicated by the fact that some exogenous antigens such as viruses from cells that have undergone lysis, and intracellular proteins from dead or dying cells can also be presented to CTL. Thus a process known as cross presentation occurs in specialised antigen presenting cells such as DCs where there is a specific pathway which delivers extracellular proteins into the MHC I presentation pathway in order to allow presentation of exogenous antigens to CTL (Lizee et al., 2003;Rodriguez et al., 1999;Ulmer et al., 1994).

1.1.2.3.7 CD8 T cells

After recognition of a presented peptide fragment in the context of MHC, provided the correct co-stimulation is present, T cells are activated. In general TCR ligation and T cell activation leads to proliferation and differentiation into either effector or memory cells. The function of CD8 T cells is detection and destruction of cells presenting abnormal peptides derived from cytoplasmic proteins. These peptides may be derived from intracellular pathogens or abnormalities due to mutations, as in cancer. These peptides are presented in the context of MHC Class I and once they are detected CTL kill infected or mutated cells by the release of granules containing perforin and other cytotoxic mediators, or Fas-Fas ligand induced apoptosis of the target cell. Cytokines released by CTL include IFNγ, which activates macrophages and acts as a "warning" to surrounding cells inducing up regulation of MHC Class I in surrounding cells to enable easier detection of infection in those too (Stout and Bottomly, 1989).

1.1.2.3.8 CD4 T cells

There are three types of CD4 T helper cells, defined as Th1, Th2 and Th0 cells (Kamogawa et al., 1993). It is thought that Th1 and Th2 cells are driven to differentiate from Th0 cells, however at which point during differentiation this becomes a permanent change is still unclear (Grogan and Locksley, 2002). Th1 cells are induced by cytokines such as IL-12 and IFNγ, and themselves produce IFNγ and IL-2. These cytokines inhibit the development of Th2 cells and are produced in response to viruses and other intracellular pathogens (Nakamura et al., 1997). Thus the cellular branch of the immune system is stimulated, activating

macrophages and providing help for the production of effector CTL responses (Machy et al., 2002). In contrast, production of cytokines such as IL-4 and IL-10 can push CD4 Th0 cells into Th2 type cells, which are associated with a humoral response. Th2 cells through production of IL-4, IL-5 and IL-10 are very important for humoral immunity, stimulating B cells and playing a central role in antibody (Ab) class switching (Stavnezer, 1996), they also fail to activate macrophages (Stout and Bottomly, 1989).

1.1.2.3.9 'Natural' T regulatory cells

There is another sub set of cells that are thought to be generated in the thymus and are characterised by a CD4+ CD25 high phenotype. These cells are known as regulatory T cells (Treg) and can suppress immune responses. Several populations of Treg have now been characterised. `Treg were first recognised as a subset of cells that could decrease the severity of autoimmune disease (Asano et al., 1996;Read et al., 2000;Sakaguchi et al., 1996). It was subsequently shown that these cells also played a role in the control of immune responses to pathogens (Suvas et al., 2003). Cell contact is required to mediate the suppression by CD25+ Treg (Nakamura et al., 2001). In addition to this subset of cells, other suppressive cells have been identified. Some of these are thought to be generated in the periphery during the course of an immune response that are antigen specific and are derived from the memory pool of cells, but they can act in a non specific manner when suppressing other responses (Akbar et al., 2003). "Classical" Treg can also induce the production of secondary Treg in the periphery which act via cytokines such as TGFβ and IL-10. These cells have been called Th3 and Tr1,

respectively. It also appears that DCs can induce the production of Treg (Levings et al., 2005;Roncarolo et al., 2003). This will be discussed further in chapter 6.

1.1.2.4 T cell memory

Once an infection has been detected by the immune system, specific cells are activated and proliferate. Thus a large expansion of antigen specific effector T cells occurs. Once the pathogen has been cleared, this primary response comes to an end and the effector cell pool contracts leaving a small population of memory cells (Badovinac et al., 2002). Unlike B cells there is no affinity maturation of the T cell response after the first encounter with antigen; however due to competition for antigen the higher affinity T cells have an increased chance of survival and therefore become dominant in the response (Blattman et al., 2000). There is evidence to suggest that memory T cells can persist for the lifetime of an organism this is shown in the case of vaccinia virus, where 90% of individuals vaccinated against smallpox had recall memory responses presumably in the absence of antigenic re-stimulation within the last 25 years (Hammarlund et al, 2003).

The current model of T cell memory subsets reflects the heterogeneity of both homing potential and function of memory T cells in the periphery. The T cell memory subsets are broadly separated into two populations termed central memory and effector memory (Geginat et al., 2003;Sallusto et al., 1999). In general, naïve cells, once they experience antigen, go from expressing the CD45 isoform; RA, to expressing the smaller isoform CD45RO which can therefore be used as a marker of memory cells (Merkenschlager et al., 1988). However in the

CD8 population differentiated memory cells may also 'revert' to expression of CD45RA which will be discussed later.

1.1.2.4.1 Central memory T cells

Central memory cells are distinct from naïve cells due to the increased sensitivity to TCR cross linking, they are therefore quicker to divide on encounter with specific antigen and due to expansion, are at higher frequencies within the T cell pool than naïve cells. Central memory cells can also differentiate into effector cells, albeit slower than effector memory cells (Sallusto et al., 1999). Central memory T cells express CD62L (L-selectin) this is in common with naïve cells; they also express the chemokine receptor CCR7 which is responsible for the central memory cells homing to lymph nodes.

1.1.2.4.2 Effector memory T cells

In contrast, effector memory cells can rapidly mature into effector cells and secrete large amounts of cytokine shortly after re-stimulation (Sallusto et al., 1999). These cells do not express CD62L or the chemokine receptor CCR7 therefore the cells do not home to lymph nodes. Some of them do however express receptors for inflammatory cytokines and chemokines. In the case of CD4 memory cells, CXCR5 (the receptor for CXCL13, a chemokine present in B cell follicles) (Moser et al, 2002) is expressed, making these cells specialised for quickly entering inflamed tissues at the start of an infection (Mackay et al., 1992).

Recent data has shown that both central and effector memory cells are generated during the same immune response -i.e. both central and effector memory T cells

are generated that either home to lymph nodes or are more activated and home to tissues (Lanzavecchia et al, 2005). Work in mice has shown that for CD8+ T cells responding to LCMV infection, memory cell precursors were present at the peak of the immune response but did not display the functional phenotype of memory cells. These functions such as self renewal, and survival were gradually acquired during pathogen clearance. It was also found that expression of the IL-7Rα chain could be used to isolate the memory precursors at early time points during the effector phase of the response and these cells could confer protection in other animals upon adoptive transfer (Kaech et al, 2003).

Another phenomenon associated with the development of memory T cells is that there appears to be the ability to imprint on to a T cell the tissue to which it should preferentially home, this has been demonstrated using DC taken from either gut or skin, to prime a T cell response and results in effector T cells with specific homing abilities (Campbell et al, 2001, Dudda et al, 2004).

1.1.2.4.3 Maintenance of T cell memory

Recent work has measured the rate of division of different memory T cell populations. This is very exciting and novel data as it is the first *in vivo* human data to be generated of this nature. The turn over of CD4+ cells was measured using heavy glucose incorporation into DNA. Effector memory cells divided at a rate of 4.7% per day, central memory cells at 1.5% per day and naïve cells at 0.2% per day. This shows that memory cells especially effector memory cells are shorter lived cells than the naïve population and therefore are actively replenished through division (Macallan et al, 2004).

There is also a debate about what mechanisms maintain T cell memory in an organism and how long this memory lasts. There is an increasing amount of evidence that suggests that IL-7 and IL-15 are important for resting homeostasis of the T cell pool (in particular CD8+ T cells) and interferons (IFN) are important for reactivation and proliferation on re-stimulation (Hamann et al., 1997;Tuma and Pamer, 2002). It appears that in CD8+ T cells IL-15 mediates proliferation and IL-7 enhances survival. In humans it has been shown that both central and effector memory T cells proliferate in response to IL-7 and IL-15 (Geginat et al, 2003). One of the main controversies in this area is whether the central and effector memory subsets are stable or whether they can feed into one another. *In vitro* experiments have shown that central memory cells in response to IL-7 and IL-15, can loose CCR7 expression and acquire tissue homing receptors thus raising the possibility that the central memory pool might divide and differentiate into effector memory T cells (Geginat et al, 2003). However more work must be done in humans to further investigate the effector/central memory T cell relationship.

1.1.2.4.4 Phenotypic markers of memory

Although the distinction between naïve and antigen experienced cells applies to both CD4+ and CD8+ T cells, in that the isoform of CD45 expressed on the surface of the cells changes form CD45RA to CD45RO, there are other phenotypic markers which can be used to follow the maturation and differentiation of memory cells. For example Appay et al and others (Posnett et al., 1999) have identified different levels of differentiation in the CD8+ T effector memory cell population by the markers CD27 and CD28, CD27+CD28+ being the early T cell pool which can

differentiate into CD27+CD28- cells, which progress further to CD27-CD28- cells. CD28- cells have been shown to be perforin positive and less sensitive to death by apoptosis (lower expression of CD95-Fas than CD28+ counterparts) (Posnett et al., 1999).

Related to this are reports that a revertant population of antigen experienced cells that re-express CD45RA is present within the T cell memory pool (Hamann et al., 1997;Dunne et al., 2002;Faint et al., 2001;Okumura et al., 1993;Wedderburn et al., 2001). Therefore it can be said that within the population of CD8+ T cells, a CD45RA+CD27-CD28- CCR7- phenotype, which correlates well with expression of perforin and high cytolytic activity with no prior re-stimulation, represent the effector CTL population (Hamann et al., 1997;Hislop et al., 2001;Tomiyama et al., 2002).

Interestingly it has been shown that in the case of these chronic infections, the effector cells described above which are present in large numbers have an inability to respond to the homeostatic cytokines IL-7 and IL-15 probably due to the fact that they do not acquire the expression of memory markers such as IL-7Rα described earlier in section 1.1.2.4.3. This may explain the inability of these cells to exist long term and also points to a fundamental difference in both phenotype and function of CD45RO+ effector memory T cells, which exist independently of antigen stimulation and chronically stimulated CD45RA revertant effector T cells, which require antigen for survival (Harari et al 2004, Wherry et al 2004, Lang et al 2005, Lanzavecchia et al 2005).

This situation is generalised for memory cells against a transient infection, compared with individual studies in chronic infections. One study has compared immunological memory to persistent viruses such as EBV, CMV and HIV. There is evidence that in these chronic situations different subsets of cells within the memory T cell population are driven to divide and differentiate in different ways, depending on the virus. Thus it was concluded that a more specific approach should be taken when analysing responses to different viruses and one should be careful of applying observations made in the case of one virus, to other immune responses to different organisms (Appay et al., 2002)

Up until recently it has been difficult to mirror the studies in T cell memory phenotype on CD8+ T cells with similar studies on CD4+ T cells. This is because many of the studies addressing the resting phenotype of memory cells have relied heavily on the use of MHC tetramers. These have been manufactured successfully for MHC I however reagents using MHC II have been more of a challenge to produce (Cameron et al., 2002). However some data have emerged showing that the progression of CD4+ memory cells through the different stages of expressing CD27 and CD28 mirrors that which is seen in the CD8+ population for the same infection (EBV). This is in contrast with the CD4+ response seen to CMV which has CD4+ T memory cells accumulating in different stages of differentiation to those seen with EBV (Amyes et al, 2003). This again confirms that the progression of cells through the phenotypes outlined above may apply differently in different types of infection each of which must be investigated individually.

1.2 Manipulating the immune system (vaccination)

1.2.1 History of manipulation of immune responses

Edward Jenner is widely considered to be the founder of modern day vaccinology. It had already been discovered that the process of variolation in which a small amount of dried material from a smallpox pustule was inoculated into a patient, caused a mild disease in most cases and protected the patient from subsequent infection with smallpox. However 3% of cases would go on to develop fatal smallpox: therefore this was not a feasible strategy for controlling disease. Jenner made the observation that during smallpox outbreaks, dairy-maids had a lower incidence of disease and that when servants milked cows with cowpox -infected udders they suffered inflamed spots on their hands. Jenner then inoculated the servants with smallpox and showed that they did not show any symptoms of the disease. Then in 1796 in a pioneering (but ethically dubious) experiment Jenner injected matter taken from the hand of a cowpox infected dairy maid into the arm of a boy named William Summers. One week later he noted that the boy was "perceptibly indisposed": however he recovered and two weeks after the initial inoculation Jenner challenged the boy with smallpox, taken from varioles on a patient with smallpox and showed that the boy had no symptoms of smallpox infection at all. Jenner called this process vaccination after the cowpox vacinnia virus. This was the birth of vaccination, although it was almost 100 years later that Louis Pasteur developed a weakened strain of chicken cholera for the inoculation of chickens. In honor of the work of Jenner he named the process vaccination. He also developed a weakened strain of rabies for vaccination of humans.

Since the first pioneering work by Jenner and Pasteur, at least nine major diseases in man have been controlled to a certain extent in humans using vaccination. Vaccination is considered to be one of the most important and most cost effective public health interventions alongside clean water. As well as the immeasurable benefit of saving lives and preventing disability, vaccination for just two common childhood diseases, measles and Haemophilus influenzae type B (HIB) is estimated to have saved the US public health authority $900 million per year since adequate coverage of the vaccines was achieved (Cutts et al., 1999;Peltola, 2000).

1.2.2 Successes of vaccination

The most obvious success of vaccination has been the worldwide eradication of Smallpox in 1979, which was estimated to have killed 500 million people worldwide in the 1900's alone (Fenner, 1998). Although the vaccine was available throughout the 1800's it was only used sporadically and it was not until the 1950's that the suggestion was made that with increased efforts smallpox could be eradicated. This was not taken seriously until 1965 when the intensified smallpox eradication programme came into effect which not only used vaccination but also incidence reporting and containment of outbreaks along with advances in vaccine delivery to achieve its goal (Mahalingam et al., 2004). In the early 1900's the BCG vaccine for tuberculosis was introduced in the West, however access was restricted to the wealthy, while the most susceptible poorer communities continued to experience outbreaks of vaccine preventable diseases. Probably the next large achievement in the West was the great reduction of poliomyelitis through development of the oral polio vaccine which due to affordability and ease of administration achieved wide coverage. However what is probably the biggest failure of vaccination to date is

that while countries with the greatest resources and political infrastructure can reduce disease burden and achieve regional elimination of the diseases, in other countries almost 2 million children die every year as a result of diseases that are preventable with the use of cheap vaccines. For example 90,000 children develop paralytic polio each year which could be prevented with an efficient vaccination programme (WHO, 2005). Table 1.1 shows a selection of vaccines introduced for use in humans over the last 200 years (Centers for Disease Control and Prevention,1999).

Date	Disease	Date	Disease
1798	Smallpox	1962	Oral Polio Vaccine (OPV)
1885	Rabies	1964	Measles
1897	Plague	1967	Mumps
1923	Diphtheria	1970	Rubella
1926	Pertussis	1981	Hepatitis B
1927	Tuberculosis (BCG)	1987	HIB conjugate
1927	Tetanus	1999	Meningococcus conjugate
1935	Yellow Fever	2000	Pneumococcal conjugate
1955	Injectable Polio Vaccine (IPV)		

Table 1.1: Dates of introduction of vaccines against a variety of diseases since Jenner's work in 1798 a selective list adapted from (Plitkin S.A and Mortimer E.A., 1994).

From the example of smallpox it is obvious that with international cooperation, as well as efficient vaccination and containment strategies, it is possible to eradicate

diseases. To this end in 1974 the WHO created the expanded programme on immunization (EPI) which identified six diseases that had affordable vaccines that would be targeted for eradication. These were tuberculosis, diphtheria, neonatal tetanus, whooping cough, polio and measles. This has resulted in many countries virtually eliminating these diseases, but it is clear that in order to completely eradicate certain diseases an international effort is required as small pockets of low vaccine coverage and high disease rates act as reservoirs that lead to constant reintroduction of disease into areas that have controlled it (WHO, 2005).

1.2.3 Problems associated with vaccine development

Vaccine development has not been without problems. Several instances of vaccination resulting in abnormal manifestations of the disease have been reported. These have mainly been caused by a vaccine primed immune response causing increased disease severity in measles (MV), dengue and respiratory syncytial virus (RSV). The manifestation of atypical measles occurred after patients were immunized with formalin inactivated measles vaccine (Fulginiti et al., 1967). This vaccine was withdrawn. Subsequent analysis on the immune pathology caused by the vaccine and infection with MV has been carried out in rhesus macaques by Diane Griffin's group. This has shown that atypical measles may be caused by the priming of a non protective Th2 biased immune response without priming for CTL (Polack et al., 2002). Antibodies are also involved and immune complex deposition at sites of infection and recruitment of eosinophils contribute to pathology contradicting previous predictions that infection could be due to low levels of anti MV fusion protein antibody (Polack et al., 1999). However the antibodies produced against MV were of low avidity and therefore had reduced

neutralising capacity. Affinity maturation did not occur in formalin inactivated vaccine recipients and the antibodies were non protective but induced complement resulting in atypical measles (Polack et al., 2002). The RSV vaccine was also formalin inactivated and the more severe form of RSV claimed the lives of two children. This has also been attributed to the induction of an inappropriate Th2 skewed immune response which contributes to the lung pathology observed (Openshaw et al., 2001). Interestingly vaccines have recently begun to be credited with non specific positive effects on children in high mortality regions. The exact mechanisms of this are still being investigated (Aaby et al., 2003;Stensballe et al., 2005).

A more recent problem has been encountered in developed countries where adverse publicity surrounding vaccines specifically the MMR (measles mumps and rubella) has reduced take up rates of the vaccine to dangerously low levels. Vaccine coverage in some areas of the UK has fallen to below 65% therefore it is highly likely that there will soon be an epidemic like that in Ireland in 2000 leading to deaths that could have been prevented (Coughlan et al., 2002). The claims surrounding the MMR have failed to be verified by many new studies however it appears the damage has been done (Chen et al., 2004;Honda et al., 2005;Miller et al., 2005;Peltola et al., 1998;Taylor et al., 1999). Although vaccine coverage rates are rising again there remains a substantial population of parents that are not willing to have their child vaccinated. This highlights the very important point that public confidence must be established in any preventative intervention or herd immunity provided by high coverage rates of the vaccine will be lost and susceptible individuals will suffer (Jansen et al., 2003).

The majority of severe childhood illness occurs within the first eighteen months of life, the so called "window of opportunity". Very young children are protected from infection by the presence of IgG antibodies that are passed from the mother across the placenta during pregnancy. This is further maintained during breast feeding, by the transfer of IgA antibodies through the breast milk. These antibodies begin to wane as the child's immune system matures and breast feeding ends; however, there is a window of time when the child may not be fully protected. This may be of increased significance as it appears that vaccine induced antibodies from the mother wane more quickly than antibodies induced by natural infection. Therefore as the proportion of mothers that had the natural infection decreases, the age at which infants loose their maternal antibodies may decrease, causing susceptibility to infection to occur at an earlier age (Redd et al., 2004).

This contributes to another factor that continues to be a problem for the efficient use and development of vaccination programmes. This is the increasing evidence that an immature immune system in very young children contributes to the lack of or inappropriate response to vaccines. Initially it was believed that persistence of maternal antibodies neutralized the vaccination challenge and therefore prevented seroconversion of the infant (Albrecht et al., 1977;Osterhaus et al., 1998). This is to a certain extent true and maternal antibodies do indeed play a role. However work by Hayley Gans and colleagues has shown using measles and mumps vaccination of 6, 9 and 12 month old children that independent of maternal antibody levels there is an age dependent deficiency of the humoral response in young compared to older infants (Gans et al., 2001;Gans et al., 1998). They subsequently showed

that there appears to be no deficiency in the cellular immune response to measles vaccine in early life and in fact maternal antibody levels have no effect on the induction of this response. These data seem to indicate that it is possible to vaccinate in the presence of maternal antibodies and induce the cellular arm of the immune response which may protect against viruses and other intracellular pathogens during the window of opportunity between the waning of maternal antibodies and the full maturation of the immune system (Gans et al., 2003). However it seems this phenomenon may be disease- and precise vaccination formulation- specific, and also may only apply to CTL responses. Other groups have shown that efficient CTL responses can be produced in early life, by human babies with HCMV (Marchant et al., 2003) and efficiently stimulated by DCs from cord blood (Salio et al., 2003). However with respect to the T helper subset of cells there are several other reports of a skewing of the infant immune response towards a Th2 rather than a Th1 phenotype which predominates in later life. Studies have shown that DCs from cord blood are intrinsically deficient in production of IL-12 which is a cytokine vital for Th1 differentiation, thus potentially skewing the immune response in early life towards Th2 (Langrish et al., 2002). This has been demonstrated functionally in studies of vaccination against polio and hepatitis B (Ota et al., 2004;Vekemans et al., 2002) when compared to responses in adults. Although this may be a normal consequence of exposure to such antigens and the conclusion that responses in early life are Th2 skewed may be a result of the influence of findings in experiments in mice and may not hold true in future human studies. However it does appear to be possible to induce Th1 responses as has been demonstrated in studies of vaccination of infants with the BCG vaccine (Marchant et al., 1999).

1.2.4 New challenges for vaccine development

Along with the development of vaccines for emerging diseases such as HIV and diseases that still cause huge morbidity and mortality such as malaria, the other major challenge is to supply and distribute the vaccines to the countries in the third world which need them most. This is of utmost importance if the WHO is to achieve the aim of complete eradication of diseases such as measles and polio. In countries where vaccination rates are high such as the USA the indigenous measles transmission has been eliminated; however, there are still intermittent outbreaks from imported cases of measles showing that in any long term goal of either eradication or regional elimination, must involve helping less developed countries to vaccinate which will in turn reduce imported cases and aid the efforts of more developed countries in controlling infectious disease (Cutts et al., 1999). To this end, there has been a huge drive recently with measles vaccination in Africa to try to halt the spread of this highly infectious disease. In southern African countries which already had good vaccination coverage, from 1996 to 2000 in an attempt to regionally eliminate measles, a catch up campaign was implemented. This targeted children from 6 months to 14 years and then was enforced by follow up campaigns every 3 years after in 9-59 month olds. The results of this were very encouraging. Reported cases fell from 60,000 in 1996 to just 117 in 2000, deaths declined from 166 to zero during the same period (Biellik et al., 2002). Therefore this shows that increased efforts combined with catch up campaigns and surveillance can have a huge impact on decreasing transmission and disease burden caused by measles (Biellik et al., 2002).

However as previously discussed it is the children under one year that are most at risk, yet routine vaccination cannot occur under 9 months of age due to the chance of vaccine failure due to maternal antibodies (Osterhaus et al., 1998). Therefore if a method of vaccination could be devised that was to bypass maternal antibodies and therefore could be given at birth to provide some protection mediated by CTL until the MMR could be administered, vaccine coverage could be even higher and the goal of measles elimination even closer. Such a vaccine could also be of importance to boost the immunity of adults as it is known that the response to the vaccine is less robust than the response to MV infection, as secondary vaccine failures have been documented where individuals responded well to the vaccine yet still contracted measles (Markowitz et al., 1990). There is also evidence that CD4+ T cell memory in vaccinated subjects decreases after 20 years however CTL levels appear to be stable over this time. The significance of this for protection is unclear (Naniche et al., 2004). Therefore there is fear that immunity in general to measles may be waning, as it is unclear as yet if vaccine induced immunity is life long (Osterhaus et al., 1998). It has been shown recently that 92% of patients vaccinated 26-33 years previously still had protective plaque reduction neutralization levels (PRN) (Dine et al., 2004).

There is a further issue concerning the measles vaccine, which being a live vaccine, requires cold storage until use. This creates a logistical problem in remote areas and inappropriate storage can lead to vaccine failure. It also significantly increases the cost per dose due to the increase in transport costs associated with cold storage. A component vaccine could potentially overcome these problems. If the immunodominant peptides within the measles virus could be identified, and a

subunit vaccine made that would be stable at room temperature, this could potentially advance the cause of measles control (Putz and Muller, 2003). Related to this is the issue of consumer confidence. It is possible that a non live vaccine would be more acceptable to parents especially in light of the recent controversy. Finally, there is the need to address the fact that in the future the eradication of measles may become a reality and therefore vaccinating with a live virus (even attenuated), may no longer be desirable or acceptable (although vaccine derived attenuated measles spreading between humans has never been reported). It is therefore believed that development of an alternative measles vaccine to the live attenuated one currently used, is an important task both because of the worldwide benefits measles eradication would bring, but also since the process of developing new vaccines should improve our knowledge of the interaction between the human immune system and this highly infectious virus (Putz et al., 2003).

Many different strategies have been employed in the search for an alternative measles vaccine. Most of the work on experimental vaccines has so far been carried out in mice and non human primates. Vaccines based on viral or bacterial vectors containing recombinant viral proteins, DNA or peptide epitopes have been developed and are reviewed extensively in (Putz et al., 2003). Single MV proteins have been used and have shown priming of antiviral CTL in mice (Etchart et al., 2001).Two different groups have published work on DNA based vaccines which protect Rhesus macaques despite the presence of neutralising MV antibodies at the time of vaccination (Polack et al., 2000;Premenko-Lanier et al., 2004). Despite encouraging early reports that DNA vaccines induced potent Th1 responses (Martinez et al., 1997), more recent studies have shown that the MV

Haemagglutinin (H) protein skews the immune response towards a Th2 response (Polack et al., 2003), obviously undesirable following the investigations into atypical measles. The MV Fusion (F) protein, despite the identical routes of administration skewed towards a Th1 response identifying the complex nature of host/ vaccine antigen interaction (Polack et al., 2003) and that a combined approach to component vaccines may give a more balanced immune protection. Other novel strategies include the expression of MV proteins in carrots which retain conformations that induce neutralising antibodies when injected. The benefits of this are two fold, firstly antigen can be produced and purified for injection in the country of distribution lowering costs and secondly as carrots can be eaten raw, if it was shown that effective immune responses could be induced to measles via the oral route of administration as with polio, both production and administration costs and safety concerns would be significantly reduced (Bouche et al., 2002;Bouche et al., 2003).

1.2.5 Peptide based vaccines

Another line of investigation into potential new vaccines uses the identification of epitopes – the consecutive polypeptide regions of a protein that are recognised by either B cells or T cells. Vaccines of this type have been shown to be effective in animal studies whether delivered as peptides or as DNA coding for peptides (Livingston et al., 2002;Vuola et al., 2005) and that multiple epitopes can be delivered at the same time without one response overwhelming the others due to immune dominance. There are several peptide based vaccines currently in trials in both animals and humans. A vaccine based on a single CTL epitope against the MV related virus RSV was shown to elicit an effective CTL response which did

protect mice from establishing RSV infection but disease may have been enhanced by the same CTL (Simmons et al., 2001). A peptide based vaccine has also been trialed in humans as a therapeutic HIV vaccine used in an attempt to boost host responses to the virus and reduce the need for antiviral therapy (Asjo et al., 2002). There has also been some success with peptide vaccines against malaria where studies have successfully shown immunogenicity of the vaccine in humans and have been both immunogenic and protective in animals (Kashala et al., 2002;Tsuji and Zavala, 2001) .

1.2.6 Identifying specific peptides

There are several different techniques that have been used to identify viral epitopes. These include initial studies with cells lines deficient in certain molecules important for the loading of peptides. MHC I surface expression is reduced in these cells lines as the MHC I is no longer stabilised at the cells surface. If exogenous peptide is added to these cells that can bind to HLA-A2*0201 the measurable MHC I on the cell surface increases. This technique has been used to identify epitopes in both influenza (Nijman et al., 1993) and EBV and HIV (Stuber et al., 1992). Other strategies have used microcapillary high performance liquid chromatography-electrospray ionization-tandem mass spectrometry to identify peptide motifs (Hunt et al., 1992) and in the specific case of measles by the same protocol comparing MV infected cells with uninfected cells. This allowed the identification of naturally processed CTL epitopes one of which proved to be immunogenic in transgenic mice (van Els et al., 2000). Another technique that Goulder et al have used to identify HIV epitopes is the use of overlapping synthetic peptides that are 15 amino acids long and overlap by 10 amino acids. These are used in pools to stimulate

peripheral blood mononuclear cells (PBMC) from patients, in this way responding 15mers can be identified and further analysed to identify the dominant sequence (Goulder et al., 2001). This is a high throughput and fast assay, which may be of great benefit when working in the field. It also requires less starting material than other methods which involve growing cell lines or bulk cultures, and which can be difficult to interpret (Rodda, 2002).

1.2.7 Measuring immune responses to infection and vaccines

The release of IFNγ is considered to be an effector function of re-activated effector CTL and occurs within hours of re-encounter with antigen. This can be measured in a variety of ways to assess CTL frequency and response to vaccines and virus antigens. This is further discussed in chapter 3. The ability to measure such responses depends on the precursor frequency of T cells specific for a certain antigen. Numbers of antigen specific cells have been measured in a variety of different infections. In order for efficient detection of responses, the assay must be sensitive enough and the precursor frequency of cells high enough to reproducibly allow measurement of responses over background. Using limiting dilution analysis and cytotoxic T cell assays one group found responses to hepatitis C virus (HCV) to be in the region of 10 responders per million PBMC compared to up to 40 responders per million PBMC against the Influenza matrix peptide (Rehermann et al., 1996). Further analysis of the influenza matrix peptide has shown that the precursor frequency to this epitope is in the region of 30 responders per million cells as shown by ELISpot (Jameson et al., 1998). However in a chronic infection such as HIV where the T cells are constantly stimulated with antigen, responses of between 75 and over 1500 responders were seen per million PBMC (Goulder et

al., 2001). This finding was verified in another chronic virus Epstein Barr Virus (EBV) where up to 2,500 responders to one peptide could be detected per million PBMC in infected patients (Tan et al., 1999).

1.2.8 Peptide epitopes in Measles

A peptide based vaccine could potentially address many of the current problems associated with MV vaccination as outlined in section 1.2.4. A vaccine which stimulated all parts of the immune system would be most desirable as antibodies are the current measure of protection although CTL and Th cells are obviously important (section 1.3). MV B cell epitopes have been identified, these are usually seen in the context of the folded protein and are therefore not consecutive peptides within the sequence of the protein however some are and have been shown to be protective in animal models (Atabani et al., 1997;El Kasmi et al., 2000). More recent work by Putz et al has identified further B cell epitopes within the MV H protein the response to which is not neutralized by MV antiserum they are currently using bacterial toxin conjugates to develop candidate vaccines (Putz and Muller, 2003).

The identification of potential MV T cell epitopes has also been a priority. Several proof-of-principle studies have found both CTL and Th epitopes in mice that when used as vaccines have proved to be protective to re-challenge with rodent adapted MV strains (Beauverger et al., 1994;Obeid et al., 1993;Schadeck et al., 1999). Despite MV being widely studied, relatively little is known about its recognition by the human immune system, and specifically the peptide epitopes that human T cells recognise. Studies have seen proliferative responses to predicted Th MV

epitopes (Hickman et al., 1997;Marttila et al., 1999;Nanan et al., 1995) and CTL MV epitopes (Nanan et al., 1995). These putative CTL epitopes were further analysed by van Els et al using microcapillary high performance liquid chromatography- electrospray ionization-tandem mass spectrometry and were not found to be epitopes processed by MV infected B cells for presentation at the cell surface (van Els et al., 2000). They did however identify 3 other epitopes one of which from the core protein was expressed at very high density (C84-92). Further analysis showed that this epitope was responded to very strongly in naturally infected children with acute infection; however responses appeared not to persist to memory (Jaye et al., 2003). These predicted epitopes are all HLA-A2*0201 restricted as this is the most common HLA-A allele, with 40% of the Caucasian population carrying it. Herberts et al identified another CTL epitope which was HLA-B*2705 restricted (Herberts et al., 2001). Despite the large amount of work involved here and the identification of several putative CTL epitopes little work has been done to examine to what extent these play a role in the human response to both natural measles infection and vaccination. The work outlined in this thesis initially attempts to further this work by investigating the T cell memory responses of immune individuals (MV seropositive) to previously identified MV CTL epitopes using the strategy used by Goulder et al to identify interferon γ (IFNγ) producing cells in the enzyme linked immunospot assay (ELISpot) in response to MV peptides (Goulder et al., 2001).

1.3 Measles virus (MV)

1.3.1 Measles Virus background

Measles is a highly communicable viral disease transmitted by aerosol from person to person with humans as its only natural reservoir. One measure of transmissibility of a virus is the R_0 value. This indicates the number of secondary cases one individual index case can cause, for measles the value is 10 one of the highest for any virus reported (Rima, 2001, Edmunds et al, 2000). Due to this high infectious nature, any vaccination strategy requires between 95 and 99% of the population to be protected in order to halt viral transmission. In most cases measles causes fever, conjunctivitis and a cough. It can be identified through the characteristic red blotchy rash (figure 1.5), which appears on the third day of illness.

Measles is frequently associated with middle ear infection or diarrhoea due to opportunistic infections that are established due to transient immunosuppression elicited by the measles virus (section 1.3.8). In 1846 Peter Panum made the important observations of the infectious nature of the virus, the 14 day incubation period from exposure to the onset of the rash and the fact that despite the lack of re-exposure after infection immunity was life long (Panum P.L., 1989- a re-publication of the original paper). This was done making observations in the Faroe Islands where measles infection had been reintroduced after 60 years. Older members of the community were still immune whereas everyone who had not been previously exposed became infected, the disease being most serious in younger children.

Figure 1.5 Child displaying the typical measles rash, kindly provided by Prof. John Stephenson (LSTHM)

1.3.2 Measles virus complications

Measles is not only responsible for the rash seen during infection. The secondary infections that occur due to the transient immune suppression elicited by MV infection can be devastating. This is best illustrated by statistics from the pre vaccination era in the USA where health care and support facilities were good. 4 million children were infected per year –essentially the whole birth cohort. This resulted in an estimated 500 deaths, 48,000 hospitalisations, 100,000 cases of otitis media often associated with hearing damage, 4,000 cases of encephalitis 1,000 cases of which resulted in permanent brain damage and 150,000 cases of respiratory complications often associated with secondary pneumococcus infection (White et al., 1985). These figures show that 10% of measles cases result in serious complications, this figure being far higher in developing countries along with the death rate in those countries estimated to be as high as 2% (MMWR, 1990;White et al., 1985). There is also a more rare complication of measles

occurring in between 1 in 100,000 and 1 in 300,000 cases (Rima, 2001). This is sub acute sclerosing pan encephalitis (SSPE). SSPE occurs on average 8 years after infection and is the result of a persistent MV infection which causes demyelination and neuronal infection. Depending on the neuronal systems infected this leads to severe and progressive neurological deficits and death within 3 to 9 months after the onset of symptoms (Rima, 2001).

1.3.3 Vaccination strategies

The majority of measles vaccines are based on the Edmonston strain of measles, so called after the patient from whom it was isolated in the 1950s, vaccine production soon followed (Katz, 1959). The Edmonston B vaccine was developed by Enders and Peebles by passaging the Edmonston strain 24 times in primary kidney cells and 28 times in human amnion cells (Enders, Peebles, 1954). The strain was then adapted to chicken embryo cells for 6 passages. This attenuated measles virus was licensed as a vaccine in the United states in 1963. This vaccine was associated with a high rate of rash and fever although the more severe symptoms of measles were avoided. It was found that co-administration of immunoglobulin reduced adverse reactions by 50% (Krugman, 1963; Stokes, 1961)

It is on this original strain that most other vaccines available today are based, having been further attenuated. Most notable of these are the Schwarz; attenuated by passaging Edmonston A in chicken embryo fibroblasts, the Moraten strain; attenuated in a similar way from Edmonston B and the Edmonston-Zagreb; derived

from Edmonston B and adapted for passage in the human diploid cell line WI-38 (Plotkin and Orenstein, Vaccines 3rd edition).

The Moraten vaccine (Attenuvax, Merck) is the only vaccine used in the United States and the Schwarz vaccine is the one that predominates elsewhere. Other vaccines not derived from Edmonston are also in use around the world including CAM-70 and TD97 isolated from Tanabe strain and used in Japan (Hirayama, 1983). The Shanghai-191 strain used for vaccination in China and the Leningrad-16 strain used in Russia and Eastern Europe (Peradze, 1983).

Since 1971 the measles vaccine has been combined with two other live virus vaccines for mumps and rubella known as the MMR vaccine although this was not implemented as a vaccine strategy until much later. This vaccine contains no less than 1000 TCID (Tissue culture infectious doses) of measles (Moraten strain in US, and Enders in UK) 5000 TCID mumps (Jeryl Lynn strain) and 1000 TCID of rubella vaccine virus (RA27/3 strain). Worldwide there are different formulations containing different MV and other virus strains depending on the strains that had been used to vaccinate in the individual vaccines. For example Japan uses the ALK-C measles strain, the Hoshino mumps strain and the Takahashi rubella virus (Plotkin and Orenstein, Vaccines 3rd edition).

Production of the different vaccine strains is in general carried out in the same way. The vaccine strain of virus is cultured in primary chick embryo cells. The cultures are inoculated with the Moraten strain of MV, and this is then incubated for several days at 32°C. The cultures are then washed to remove FBS and the medium

replaced with one containing neomycin, sucrose, buffer, amino acids and human albumin. While cells are maintained at the same temperature, medium containing virus can be removed periodically. This medium is frozen and viral titre is determined. Once quality checks have been completed the frozen cell supernatants are dispensed into vials at the correct dose and lyophilised. To stabilise the formulation at this stage, sorbitol and hydrolised gelatine are added. This lyophilised product is then reconstituted with sterile distilled water immediately prior to injection (Elliott, 1984).

Before measles vaccination was introduced in the 1960's (USA) and 1980's (UK) measles epidemics occurred regularly every 2-5 years. Crowded urban settings are ideal for outbreaks and the larger the community the shorter the time between epidemics. The success of vaccination strategies in developed countries such as the USA is impressive, with a reduction in measles cases by 99% between 1963 and 1989 (Markowitz et al., 1989). However as discussed in section 1.2.4 although the MV vaccination programme has been one of the most successful and economic, there is still the need to both increase current efforts especially in developing countries, and increase research into the immunology of measles if eradication of this disease is to be achieved.

As outlined in section 1.2.4 the reason that the advent of the measles vaccine has not had as significant an effect in developing countries, (aside from some areas with low vaccine coverage and potential vaccine storage problems) is the age at which children are most susceptible to MV infection. This is around 9 months of age. Vaccinating this early can mean failure of the vaccine due to maternal

antibodies (Markowitz et al., 1990). Since T cell mediated immunity plays a central role in clearance and protection from MV, an ideal solution to this would be the development of a vaccine that stimulated cellular immunity possibly via a combined peptide approach thus bypassing maternal antibodies and potentially allowing vaccination at birth. A peptide subunit vaccine would be ideal for this function as discussed in section 1.2.5.

1.3.4 MV structure

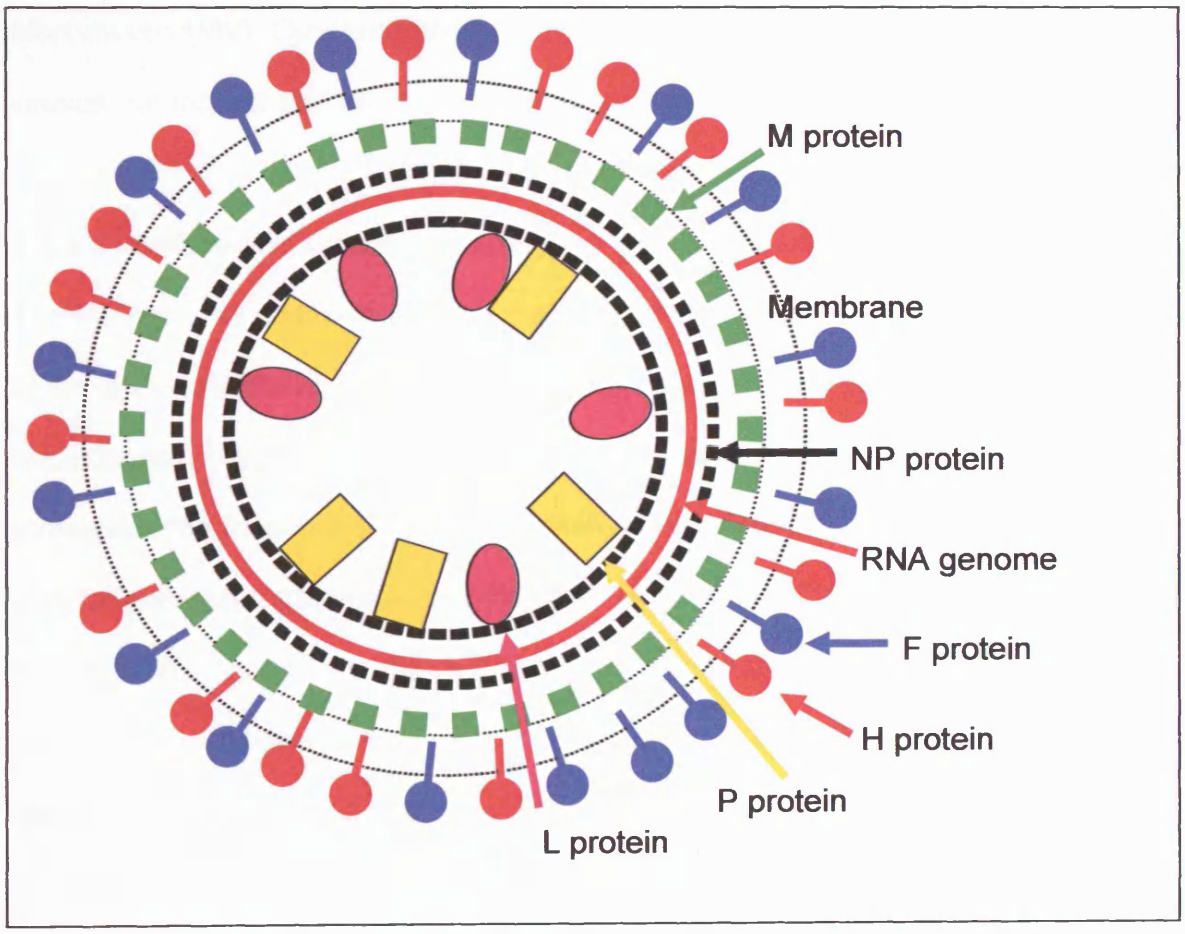


Figure 1.6: Diagram of the measles virus showing the major structural proteins and the RNA genome protected by the nuclear protein. H-Haemagglutinin, F-Fusion, L-Large, M-Matrix, NP- Nucleoprotein, P-phosphoprotein.

Measles virus (MV) is a large enveloped single stranded negative sense RNA virus and is approximately 100-250nm in diameter (figure 1.6). The MV genome is approximately 16Kb in size which is divided into six transcription units separated by 3 nucleotide long intergenic sequences (Rima, 2001). MV is a member of the *Paramyxoviridae* family. This family is divided into two subfamilies known as the *Pneumovirinae* of which the human pathogen RSV is a member and *Paramyxovirinae* (Bellini et al., 1994). Within the *Paramyxovirinae* are three genera, *Respirovirus* (human parainfluenza 1 and 3), *Rubulavirus* (Mumps) and *Morbillivirus* (MV). Other members of the *Morbillivirus* genus include the animal viruses, rinderpest and canine distemper virus (Rima, 2001).

1.3.5 Structure and function of measles virus proteins

The MV genome codes for at least eight different proteins. The MV virion consists of six of these proteins. There is an outer lipoprotein envelope and an internal nucleocapsid (figure 1.6). Three viral proteins are associated with each of these structures, the Fusion (F), Haemagglutinin (H) and Matrix (M) proteins associate with the envelope and the Nucleo (NP), phospho (P) and large (L) protein protect the 16Kb RNA genome in the nucleocapsid (Schneider-Schaulies and Meulen, 2002). Other proteins include the Core (C) protein and V (V) proteins which are generated from the gene encoding the P protein but have unknown non structural functions although are thought to play a role in virulence as further discussed in section 1.3.5.4.

1.3.5.1 Haemagglutinin (H)

The MV H protein is the protein which mediates binding of the virion to the cell surface prior to entry into the cell. It is a 617 amino acid long, type 2 membrane glycoprotein, and is positioned protruding from the envelope along with the F protein, forming the spikes. It is thought that it is present as a homotetramer in the virion (Ogura et al., 1990). In order for the virus to enter the cell, H protein must bind to the host cell but also be free to interact with the F protein as this is critical for cell fusion. This has been shown by mapping binding sites of neutralising antibodies (Wild et al., 1991). It has also been shown that the H protein is important for tropism of the virus to different cells types. This is presumably due to the different receptors (see section 1.3.6) that H can use on the cell surface, and the affinity of the H in the different MV strains for receptors preferentially expressed on certain cell types (section1.3.6 and figure 1.7). Another difference between the wild type and vaccine strains of virus is the ability of the H protein to activate cells via TLR2. The wild type strains can do this yet it appears that the mutation that prevents this in vaccine strains is the same mutation that allows vaccine strains to bind to CD46. Consequences of TLR2 ligation include the up-regulation of CD150 which may be another mechanism by which the wild type strains have increased viral spread-by up-regulation of the receptor required for cell entry (Bieback et al., 2002)

1.3.5.2 Fusion (F)

The MV fusion protein is a 550 amino acid long, type 1 membrane protein, that as its name would suggest is responsible for fusion of host cell and virion membranes. This process occurs at neutral pH and results in the release of the viral capsid into

the cytoplasm of the cell. The F protein is synthesised in the cell as a precursor protein F0 this is the cleaved and therefore activated into the F1-F2 heterodimer. This becomes a homotrimer of the F1-F2 dimer on the surface of the virion. When the H protein binds to its receptor a conformational change is induced in the F protein leading to insertion of the hydrophobic fusion domain of the protein into the target cell membrane (Wild et al., 1994). The MV F protein is also responsible for some of the immunosuppression seen in MV infection. This process does not require cell fusion and merely requires the presence of the F1-F2 heterodimer. This can either be on the virion itself or on the surface of an infected cell, thus suppressing the proliferation of T cells that may have recognised MV antigen on the cell surface (Weidmann et al., 2000).

1.3.5.3 Nucleoprotein (N)

The MV NP protein is a 525 amino acid long phosphoprotein. It binds to the RNA in the virion protecting it from degradation and aiding in its replication once inside the cell (Zhang et al., 2002;Zhong et al., 1999). The NP protein has been shown to have immunoregulatory functions in its own right, aside from the functions in replication and maintenance of the RNA genome of the virus. MV NP has been shown in mice to directly inhibit IL-12 production by DCs (Marie et al., 2001) through direct binding to the FcγRII on the surface of cells. Through the same interaction, NP can also bind human B cells but not T cells, and this interaction reduced production of CD40 by 50% thus inhibiting the production of antibodies and contributing to immunosuppression (Ravanel et al., 1997). The functional domain of the protein has been mapped to the C terminus and had been found to bind a variety of cells via a different and as yet un-characterised receptor. The

result of this is the arrest of those cells in the G0 phase of the cell cycle and thus suppression of proliferation (Laine et al., 2003). Not only is NP derived from lysed cells available to act in this way but it also appears to enter the late endocytic compartment, couple with the FCγR and be expressed on the surface of infected cells, and be secreted into the extracellular compartment to interact with uninfected cells (Marie et al., 2004). However, despite the profound suppressive ability of NP, Etchart et al have shown that NP can be immunogenic and can recruit DCs to the site of injection and prime CTL when used as a vaccine antigen (Etchart et al., 2001).

1.3.5.4 Other MV proteins

The P (507aa) and L proteins (2183aa) are known to combine with the NP protein to form the nucleocapsid. These two proteins are also thought to form the RNA polymerase which is required for the replication of the genome and enabling mRNA to be made and the viral proteins produced in the host cell (Hamaguchi et al., 1983). The M (335aa) protein is involved in the coordination of assembly of the new virions from infected host cells. A MV mutant lacking M also has an increased ability to promote cell fusion, this may be a feature of strains that cause SSPE, allowing the MV strains defective for M to penetrate further into the brain (Cathomen et al., 1998). The V (300aa) and C (186aa) non structural proteins are thought to act as virulence factors. Studies in transgenic mice expressing CD46 which allow them to become infected with MV (see section 1.3.6), have shown that although equally immunogenic, MV strains lacking V or C proteins have reduced spread and pathogenesis in the mice, with viral load significantly reduced (Patterson et al., 2000). The V protein has also been shown to inhibit STAT1 and

STAT2 phosphorylation in human cell lines which resulted in reduced expression of type 1 IFN, contributing to the immunosuppression elicited by MV (Takeuchi et al., 2003). The C protein was also found to be necessary for viral replication in human PBMC but not in vero cells where as the V protein was not necessary in either setting for viral replication to occur. Both defective viruses efficiently inhibited T cell proliferation to the same level as wild type virus therefore the C and V proteins are not necessary for the immunosuppressive effect (Escoffier et al., 1999)

1.3.6 Receptor binding and entry to cells

The vaccine strains of MV stimulate an effective immune response in vaccinated individuals, however these strains are significantly attenuated compared to wild type strains, indicated by the absence of most of the clinical symptoms caused by wild type MV infection. Several studies have investigated the genomic differences between wild type and attenuated MV strains and have found variation in all of the major proteins (NP, P, M, F, H and L) (Bankamp et al., 1999;Parks et al., 2001;Takeda et al., 1999). As discussed in section 1.3.5, mutations in the H and F proteins may account for the differences in virulence between the strains and the lack of V and C proteins has been shown to inhibit virus spread in mice. Therefore mutations inhibiting the functions of these proteins could play a role in reduced pathogenesis.

The differences in the immune response between circulating wild type virus strains and the Edmonston vaccine strain could be due to mutations that have been driven by the passage of virus in non human cell lines such as chicken embryo fibroblasts and dog and sheep kidney cells (Parks et al., 2001). Wild type MV enters cells

through binding to its receptor, signalling lymphocyte activation molecule (SLAM) or CD150 (Erlenhoefer et al., 2001;Hashimoto et al., 2002). This is different to the Edmonston strain which preferentially enters via CD46 but can also use CD150 (figure 1.7). Work in the cotton rat model has identified a point mutation in the H protein that allows MV strains carrying it to enter the cell via CD46. The same study found that viruses using exclusively CD150 caused strong immunosuppression and had increased spread than strains using CD46 (Pfeuffer et al., 2003).

CD150 is a CD2 family member, a defining characteristic of which is the presence of ITSM (immunoreceptor tyrosine based switch motif) on the cytoplasmic tail. This enables these molecules to be regulated by small adapter proteins within the cell and ligation of CD150 can mediate different effects depending on the availability of the different downstream effector molecules. One of the key functions of ligation of CD150 is the production of IFNγ: several viruses have ways to subvert the function of CD150 (Sidorenko and Clark, 2003). Studies have found that despite this the contact mediated suppression of T cell proliferation seen in MV infection is not due to the down regulation of CD150 or CD46 (Erlenhoefer et al., 2001). CD46 is a cell surface glycoprotein that was first identified as a member of the regulation of complement activation family of molecules. These molecules bind activated complement components C3b and C4b preventing deposition on the cell surface and therefore complement lysis of the cell. The Edmonston strain can down regulate CD46 from the host cell surface post infection, which can lead to increased susceptibility of the cells to complement lysis thus reducing viral

replication and spread in the host (Manchester et al., 2000;Schneider-Schaulies et al., 1995).

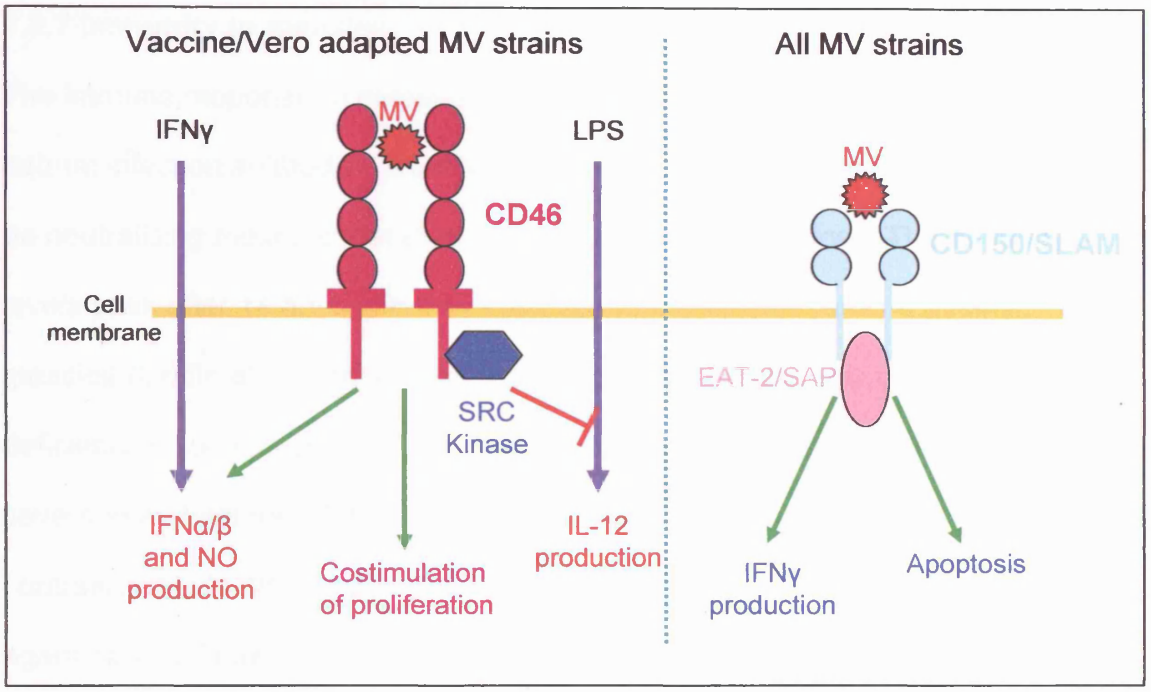


Figure 1.7: Binding of MV to receptors CD150 (all strains) and CD46 (vaccine strains) results in MV fusion and entry into target cell. CD150 is expressed on activated lymphocytes, NK cells and mature DCs where as CD46 is expressed on all nucleated cells. Molecules known to be involved in signalling are shown. Tyrosine phosphorylation of the CD46 cytoplasmic domain occurs in a SRC kinase dependent manner in response to MV infection. Signalling after CD150 ligation is dependent on SAP (SLAM associated molecule) in T and NK cells and EAT-2 in APC. Consequences of ligation of receptors are shown at the bottom of the figure with the effects proven to also occur in MV infection shown in red. Adapted from (Schneider-Schaulies and Meulen, 2002)

Interestingly recent studies have found that wild type MV can infect cells that do not express CD150 and this is not blocked using anti CD46 antibodies proving that there are as yet other unknown receptors for MV (Hashimoto et al., 2002). It has also been recently documented that wild type virus but not the vaccine strain activated Toll like receptor 2 (TLR2) on DCs via the H protein resulting in production of IL-6 in response to viral infection. This provides a mechanism by

which the immune response to the two strains differs in the stimulation of response (Bieback et al., 2002).

1.3.7 Immunity to measles

The immune response to measles consists of both B and T cell responses. After natural infection antibodies are made against N, F, H and M proteins. These can be neutralising meaning that they render the virus un-infectious. The antibody levels peak after 14 days and they correlate with clinical protection against measles (Griffin et al., 1994). Interestingly patients with cellular immune deficiencies such as severe combined immunodeficiency (SCID) are susceptible to severe even fatal measles infection as a result of giant cell pneumonia. However in contrast patients with an antibody deficiency such as X-linked agammaglobulinaemia (XLA) can still develop protective immunity to measles (Burnet, 1968). Thus as mentioned previously T cell responses may well be central to protective immunity to MV.

Natural MV infection and vaccination both lead to CD4 and CD8 T cell responses (Jaye et al., 1998a). Several groups have documented the importance of the CD8 T cell response to MV infection. Large expansions of CD8+ CTL clones could be detected up to a month after viral infection (Mongkolsapaya et al., 1999). The same was found in a larger study of children in the Gambia which also showed that both CD4+ and CD8+ T cells play a role in CTL mediated killing (Jaye et al., 1998a). More recently work in Rhesus monkeys showed that disease was much worse and lasted longer in CD8+ cell depleted animals. This correlated with a marked increase in viral titre again showing the importance of CD8+ T cells controlling viral

replication (Permar et al., 2003). The same group went on to show that depletion of B cells prior to infection had no effect on the rate of clearance of the virus and did not produce any unusual symptoms. It would be interesting to investigate in this model the consequences of re-challenge in these animals, comparing animals who had a B cell response on primary infection with those who did not in order to investigate the roles of CTL and antibodies in protection from re-infection (Permar et al., 2004). Another useful model that has been developed is a transgenic mouse expressing human CD8 and MHC I molecules. This model was shown to develop CTL with the same specificity for a HLA-B27 epitope as humans. If this could be adapted for HLA-A2 and other common human HLA alleles it may be an even more useful tool to identify MV specific epitopes (Tishon et al., 2000). T helper cells have also been shown to be involved in the response to MV infection. Moss et al demonstrated early type 1 responses in T cells with IFNγ production followed by a switch to IL-4 and IL-13 in later responses, they also showed a prolonged production of IL-10 in the plasma of infected individuals which may contribute to the immune suppression seen after MV infection (Moss et al., 2002). Another study also showed Th1 type cytokine production in immune donors to MV antigens *in vitro* (Howe et al., 2005). ELISpot and intracellular staining techniques have also showed T cell responses to measles infected B cell lines with IFNγ production (Nanan et al., 2000). It is clear that Th responses are important for both antibody production and CTL generation in response to MV. Proliferative responses and cytokine production by T cells was shown to correlate with the amount of antibody made in response to MV vaccination (Ovsyannikova et al., 2003). The same group has also shown an association between MHC II alleles and the failure to respond to vaccination against MV (St Sauver et al., 2003).

1.3.8 Measles virus immunosuppression

As previously discussed despite the success of vaccination programmes, measles is still the leading cause of vaccine preventable deaths, causing almost 1 million deaths annually (WHO, 2002). Pneumonia accounts for 60% of measles related deaths, as it is the transient immunosuppression elicited by measles that allows secondary opportunistic infections to establish themselves. The immunosuppression elicited by MV is paradoxical in that while a successful immune response is mounted to clear the infection there is a marked transient decrease in cellular immune responses as demonstrated by reduced delayed-type hypersensitivity reactions (Pabst et al., 1997;Von Pirquet C., 1908) and reduced cytokine production by, and proliferation of lymphocytes (Atabani et al., 2001;Schneider-Schaulies and Meulen, 1998). This leads to increased susceptibility to bacterial infections. This has been tested in transgenic CD46 mice infected with MV and then challenged with *Listeria monocytogenes.* Compared to mice that were not infected with MV, the MV infected mice had increased susceptibility to infection with *Listeria monocytogenes,* had a decrease in macrophage and neutrophil counts and a decrease in IFNγ production and proliferation in T cell subsets. This was not due to increased apoptosis but a decrease in proliferation in both adaptive and innate arms of the immune system (Slifka et al., 2003).

The mechanism of this immunosuppression is still debated; however it appears likely that there may be several different pathways and individual MV proteins that play a role (Moss et al., 2004). Suppression of IL-12 production by MV has been

reported up to several months after infection in humans *in vivo* (Atabani et al., 2001) and in response to LPS stimulation *in vitro* (Karp et al., 1996). However the ability of DCs to produce type 1 IFN and mature in response to MV infection remains intact (Klagge et al., 2000). This reduction in IL-12 production could in some part caused by the increased production of IL-10 (Moss et al., 2002) which may be produced by the induction of T regulatory type 1 (Tr1) cells via activation of CD4+ cells through CD3 and CD46 (Kemper et al., 2003), but this suppression of IL-12 production is also via the direct action of individual proteins. As discussed in section 1.3.5.3, MV NP has been shown in mice to directly inhibit IL-12 production by DCs (Marie et al., 2001) and is implicated in several other mechanisms of immune modulation including direct inhibition of antibody production by B cells and contact dependent inhibition of cell proliferation through cell cycle arrest (Laine et al., 2003;Marie et al., 2004;Ravanel et al., 1997). There is also a suppressive effect mediated by the MV glycoproteins H and F which as outlined in section 1.3.5.1 act together to induce a state of unresponsiveness in responder cells (Schlender et al., 1996) or apoptosis of bystander cells (Vuorinen et al., 2003). This is thought to be via disruption of the Akt kinase signalling pathway (Avota et al., 2001). Expression of these MV glycoproteins on the surface of infected DCs suppresses proliferation of T cells despite a mature DC phenotype (Dubois et al., 2001;Klagge et al., 2000). There also are non specific effects of immune modulation by MV. The MV infection causes changes in the intra cellular expression of type 1 IFN inducible protein and Hsp90 resulting in presentation of self epitopes and activation of autoreactive T cells in acute MV infection. MV infection of DCs can also induce Fas mediated apoptosis thus further disabling the immune response (Servet-Delprat et al., 2000a) and it has also been reported that both DCs and T cells in human co

cultures undergo massive apoptosis after exposure to MV (Fugier-Vivier et al., 1997). These diverse immunosuppressive effects of whole MV on human DC make it difficult to work with both live MV and individual proteins to study T cell responses *in vitro*, as proliferative responses may be hard to detect in light of the suppressive ability of the virus. In particular the apoptotic effect both wild type and attenuated MV has on DCs is of concern when attempting to present naturally processed MV antigen. This will be further discussed in chapter 4.

1.4 Project aims

The aim of this study was to characterise T cell epitopes in the MV, by demonstrating IFNγ production in response to measles peptides in immune donors and then in recently vaccinated adult volunteers. Peptides were initially chosen from reported epitopes for HLA-A2*0201 which had stimulated proliferation of T cell lines and been naturally processed but no direct ex vivo quantitation of responses had taken place (chapter 3). If this was successful it could then be used to look at responses in recently vaccinated children. In order to further map the T cell response to MV a series of overlapping peptides, spanning the different MV proteins were used in immune donors and recently vaccinated individuals in order to identify specific peptides that would warrant further investigation (Chapter 3 and 4). This approach has been successfully used to map the major T cell epitopes of HIV (Goulder et al., 2001).

When studies in chapters 3 and 4 were inconclusive, a system was developed by which individual MV proteins could be delivered to DCs with the use of adenoviral vectors this was to allow processing and presentation of MV proteins which would

more closely mimic the route of natural infection and avoid the cytopathic effects that MV has on antigen presenting cells. DCs infected with adenoviral vectors containing the coding sequences for MV proteins would then be used to stimulate autologous T cells in order to grow short term T cell lines. This would significantly increase the precursor frequency of antigen specific T cells, which were re-stimulated in the ELISpot assay with peptide pools specific for the protein to which the T cell line had been grown, in order to identify MV specific epitopes.

During the course of these experiments, an interesting observation was made. T cells proliferating in the presence of adenoviral infected DCs had reduced proliferation when compared to T cells proliferating in the presence of mock infected DCs. This phenomenon was further investigated, firstly to establish if the effect was due to an adenoviral induced defect in the antigen presentation capacity of the DCs or if the effect was due to an active suppression by the adenoviral infected DCs. Supernatant transfer, transwell and cytokine blocking experiments were used in order to determine a mechanism for the observed suppression of proliferation. This work is detailed in chapter 6.

During the course of this project, the opportunity arose to utilise the same methods used throughout the other sections in order to investigate the nature of the T cell response to a different pathogen. This was *N. Meningitidis B*. The aim of this section of the work was to characterise the T cell response in individuals vaccinated with an outer membrane vesicle (OMV) based vaccine and to determine the nature of this response. This was done using methods that had been shown to be effective in other studies such as proliferation assays, and also

ELISpots to detect both IFNγ and IL-5 release in response to re-stimulation of PBMC from vaccinated individuals with OMV. This section of the work is discussed in chapter 7.

Chapter 2 General materials and methods

All cell culture work was performed using endotoxin free sterile reagents, in a Gelaire® BSB 4A Category 2 fume hood, local safety regulations were followed at all times.

2.1 Volunteers

2.1.1 Vaccination study volunteers

Full informed consent was obtained from all individuals who took part in the studies. For both the Meningitis B and the MMR vaccine trials, healthy adult volunteers were selected from the Institute of Child Health, London. All studies had full ethical approval from the Great Ormond Street Hospital/ Institute of Child Health regulatory ethics committee.

2.1.2 Other volunteers

Healthy volunteers for other parts of this project were selected from within the Immunobiology, Rheumatology and Molecular Immunology units at the Institute of Child Health, London. On recruitment these individuals were assigned an AW (Adult Well) number and were typed for the presence or absence of HLA-A2 protein expression (see section 2.5.7).

2.2 Blood Samples

2.2.1 Preparation of Peripheral Blood Mononuclear Cells (PBMC)

Blood samples were collected into universals containing 35U of preservative free heparin (CP pharmaceuticals Ltd. Wrexham) and processed within 6 hours of collection. PBMCs were isolated from venous blood by Lymphoprep^{TM} (Axis-Shield,

Nycomed, Oslo, Norway) gradient centrifugation. Whole blood was diluted with and equal volume of RPMI 1640 medium (Life technologies, Paisley, Scotland) supplemented with 2mM L-glutamine (Life technologies) and Penicillin/Streptomycin ($100Uml^{-1}/100\mu gml^{-1}$ conc.) (Life technologies)(RPMI). Half the final blood volume of Lymphoprep™ was put in a falcon tube and the blood was layered on top using a pipette. The blood samples were spun at 800g for 30mins in either an IEC Centra-8R centrifuge or a Beckman Coulter, Allegra™ 21R centrifuge with the acceleration and deceleration low. The resulting interface layer of PBMCs was removed with a pastette into a clean falcon tube. The volume was then at least doubled with RPMI and spun again at 500g for 10 minutes. The supernatant was removed and the cells re-suspended in 50µl RPMI by gentle flicking then made up to the required volume for counting with RPMI (usually the same volume of blood originally taken). The cells were then spun again at 300g for 10 minutes and re-suspended in the desired volume of either complete medium (CM) (RPMI 10% v/v foetal calf serum (FCS) (Insight Biotechnology), freezing medium if to be frozen (see 2.2.4) or RPMI 5% AB human serum (Sigma, St Louis, Missouri, USA) for all DC work.

2.2.2 Preparation of serum samples

For some volunteers 1ml of blood was taken for storage as serum to look at antibody and cytokine levels at a later date. This involved 1ml blood incubated in a serum tube with no anti-coagulant in a 37°C water bath for 30 minutes. After clot formation, the liquid fraction was spun at 10000rpm to remove any cells and the supernatant removed before being stored in 200µl aliquots at –70 °C.

2.2.3 Counting viable cells

10μl of cell suspension was added to 10μl of trypan blue 0.4% (Sigma) in a U bottomed plate well for 1 minute. 10μl of this was then placed on to a Neubauer counting chamber and counted under a microscope. All unstained, therefore live cells were counted in the specified 25-box field. Blue cells were noted but not counted and cells were routinely > 97% viable. The total number of cells was then calculated by multiplying by 2 to account for the trypan blue dilution and then by 10^4 to calculate the number of cells per ml.

2.2.4 Freezing cells

All cells were cryo-preserved in liquid nitrogen for long-term storage. After counting cells were spun and re-suspended at 10 x 10^6 cells per ml in freezing medium (FM), FCS supplemented with 10% v/v Dimethyl sulphoxide (DMSO) (Sigma). Between 0.5 and 1ml of this solution was then transferred in to cryovials (NUNC, Roskilde, Denmark) and these were placed into a freezing pot with isopropanol coolant then placed at -70°C (thus cooling at 1°C per minute) for 24hours before being transferred to liquid nitrogen. The only exception to this was freezing the 293 cells and Thp1 cells, which are sensitive to DMSO. In this case glycerol was used in the place of DMSO in the FM.

2.2.5 Thawing cells from liquid nitrogen

Cryo-preserved cells were removed from liquid nitrogen and rapidly thawed in a 37°C water bath. Cells were then transferred to 2ml of pre warmed 37°C FCS in a

drop wise fashion. The universal was then filled up with warmed medium and spun at 300g for 10 minutes before being counted.

2.3 Summary of cells and culture medium

2.3.1 Summary of different cells and culture medium

A summary table of the different cells and culture medium used throughout this project is outlined in table 2.1. All reagents are from GIBCO BRL unless otherwise stated. Penicillin at 100Uml^{-1}, Streptomycin at 100μgml^{-1} and 2mM L-glutamine was added to all media and is indicated below as P/S/glu. Foetal Calf Serum (FCS) (Insight Biotechnology, lot no A01120-650) throughout the project was the same lot number that had been batch tested previously.

2.3.2 Cell line culture

Cell lines were routinely thawed into and cultured in a T75 flask (Corning Life Sciences, UK) at 37°C 5% CO_2 in a humidified incubator (Jencons-PLS, Beds, UK) then transferred after 24 hours to a T225 (Corning Life Sciences) for further amplification prior to use. The same incubator was used throughout the project.

2.3.3 Splitting adherent cell lines

All adherent cell lines were grown to approximately 90% confluence before being split. This was done by removing the old medium and washing gently twice with PBS Dulbeccos (Life Technologies) then adding enough 37°C Trypsin/EDTA (Gibco) to cover the monolayer of cells. The flask was then placed back into the 37°C incubator for 5 minutes or until the monolayer had floated off. 20ml of

medium was then added to halt the reaction and the cells were spun at 300g for 10 mins then re-suspended in the appropriate amount of fresh medium and transferred to new culture flasks.

Cell type	Medium	Supplements	Origin	Use in project	Comments	Publications
PBMC	RPMI 1640	P/S/glu 10% FCS	Human volunteers	Ex vivo assays for memory to antigen	Media called CM in further methods	-
PBMC	RPMI 1640	P/S/glu 5% Human AB serum (Sigma)	Human volunteers	Growing Dendritic cells (DC)		-
293	MEM	P/S/glu 20mM HEPES, 1% 100x ne aa (Sigma)	Human embryonal kidney	Permissive cell line for amplifying viral stocks	Freeze in FCS. 10% glycerol	(Fooks et al., 1995a)
Vero	DMEM	P/S/glu 10% FCS	African Monkey Kidney	Cell line for growing and titrating MV		(Prasad and Mnere, 1980)
U937	RPMI 1640	P/S/glu 10% FCS	Human histiocytic lymphoma	Monocytic cell line for optimising DC protocol	Exhibits early monocytic phenotype	(Sundstrom and Nilsson, 1976)
Thp1	RPMI 1640	P/S/glu 10% FCS	Human monocytic leukaemia	Monocytic cell line for optimising DC methodology	Start up in 15% FCS Freeze in 10% glycerol	(Tsuchiya et al., 1980)
T2	RPMI 1640	P/S/glu 10% FCS	Fusion of .174 B cell line and CEM T cell line	TAP deficient line used as APC in ELISpots	-	(Zweerink et al., 1993a)
K562	RPMI 1640	P/S/glu 10% FCS	Human Lymphoma	Original cell line, used as control in ELISpots for K562-A2	Low MHC class I expression used as NK cell target	(Andersson et al., 1979b)
K562-A2	RPMI 1640	P/S/glu 10% FCS 10mM HEPES	Human Lymphoma transfected with human HLA-A2*02	APC in ELIspots	Keep at low passage to avoid loosing A2 expression	(Britten et al., 2002b)

Table 2.1 Summary of cell lines and media used in project

2.4 Enzyme Linked Immuno-spot assays (ELISpots)

2.4.1 IFNγ ELISpot

96 well polyvinylidine diflouride (PVDF) (Millipore, Bedford, MA, USA) plates were coated for 2 hours at 37°C/5%CO_2 with 50μl per well of 15μgml^{-1} anti-human IFNγ mouse IgG1 (clone D1K, Mabtech, Sweden). This was diluted in PBS (w/o Mg and $CaHCO_3$, GIBCO™, USA) from the stock. Following incubation the wells were washed x8 in 200μl PBS 1% FCS in sterile conditions using a vacuum pump to aspirate (Vaccubrand GmbH, Wertheim, Germany). 100μl complete medium (CM) (RPMI plus 10% v/v FCS) was then added to each well and incubated for 1hour at 37°C/5%CO_2 to block non specific binding sites. This medium was aspirated out and PMBC (either fresh or thawed) were re-suspended at either 1 or 2.5 x 10^6 ml^{-1} in CM. 100μl was transferred to each well (i.e. 1 or 2.5 x 10^5 cells per well). 100μl of antigen was diluted in CM to 2x final concentration and added to the relevant wells in either triplicate or quadruplicate. If peptide pulsed antigen presenting cells were being used these were pulsed for 3 hours with peptide at a concentration of 10μM, washed and re-suspended and added per well in an APC: PBMC ratio of 1:4. The plates were then incubated for 16 hours at 37°C/5%CO_2. Following incubation the cells were discarded and wells were washed x8 in PBS as before. 50μl of 1μgml^{-1} biotinylated mouse IgG1 anti human IFNγ (clone 7-$B6^{-1}$, Mabtech) was transferred to each well and the plate was incubated for 2 hours at 37°C/5%CO_2. The plate was washed x8 with PBS and 50μl of 1μgml^{-1} streptavidin alkaline phosphatase (Sigma) added to each well. The plate was incubated again for 1 hour at 37°C/5%CO_2 and was washed again x8 with PBS. For detection of cytokine production the wells were developed using 5-bromo-4-chloro-3-

indolyphosphate and nitroblue tetrazolium (BCIP/NBT) substrate (BioRad, Hemel Hempstead, UK). This was combined just prior to use as per the manufacturer's instruction and diluted in substrate buffer. 100μl of this was added per well and incubated at room temperature for 5 minutes or until dark blue spots appeared in the positive control wells. Running the plate under tap water for 3 minutes stopped the development of colour.

2.4.2 IL-5 ELISpot

The protocol was the same as for the IFNγ ELISpot with the following exceptions. PVDF plates were coated with rat anti human/mouse IL-5 (clone TRFK5, Pharmingen) at a concentration of $10\mu gml^{-1}$ overnight at 4°C. Once the cells were added the plate was incubated at 37°C/5%CO_2 for 48hours. The secondary antibody biotinylated rat anti human/mouse IL-5 (clone JES1-5A10 Pharmingen) was added at a concentration of $1\mu gml^{-1}$.

2.4.3 IL-10/ IL-12 ELISpot

The protocol is as for IFNγ ELISpot with the following exceptions. PVDF wells were coated with rat IgG1 anti human IL-10 (Clone 9D7 Mabtech) or mouse IgG1 anti human IL-12 (code IL-12 I Mabtech) at $10\mu gml^{-1}$ for 2 hours at 37°C/5%CO_2. On addition of the cells the plate was incubated for 24 hours before development. The secondary antibodies were either rat IgG2a biotinylated anti human IL-10 (clone 12G8 Mabtech) or Mouse IgG1 biotinylated anti human IL-12 (antibody code IL-12 II Mabtech) used at a concentration of $1\mu gml^{-1}$ and incubated overnight at 4°C.

2.4.4 ELISpot positive controls

2.4.4.1 Phorbol 12-Myristate 13-actetate (PMA)

PMA (Sigma) is a Phorbol ester and a specific activator of protein kinase C. Stocks of PMA were prepared in ethanol and stored at $1mgml^{-1}$ at -20°C. PMA was used at a final concentration of $1\mu gml^{-1}$.

2.4.4.2 Lipopolysaccharide LPS

LPS (Sigma) was stored in RPMI at a concentration of $1mgml^{-1}$ at 4°C and used at a final concentration of $100ngml^{-1}$ unless otherwise stated.

2.4.4.3 Dust mite antigen

As a positive control for the IL-5 ELISpot plate (since not every donor responded to LPS), PBMC from an atopic donor were used on each plate. This donor was known to be highly allergic to dust mite and so would produce high levels of IL-5 in response to the *Dermatophagoides Pteronyssinus* antigen (Aquagen, ALK-ABELLO, Denmark) used at a final concentration of 100 $SQ\text{-}Uml^{-1}$.

2.4.5 ELISpot data analysis

After air-drying the developed plates over night at room temperature, the spots were counted using a video analyser (BioReader 3000, BioSys, GmbH, Karben, Germany). Pre-optimised reading methods, created for the project using the BioSys software were utilised. Each triplicate or quadruplicate was read and the mean number of spots determined per condition. The mean background value in control wells was either included on the graphical representation of the results, or

when comparing samples from different time points, was deducted from the mean test well values.

2.5 Fluorescence Activated Cell Sorting (FACS)

All antibody staining was carried out on ice, using cold reagents and wash buffers.

2.5.1 Buffers and solutions

FACS Buffer is PBS 1% FCS and 0.1% sodium azide (Sigma).

FIX Buffer is FACS buffer containing 1% formaldehyde (Sigma).

PERM Buffer is FACS buffer containing 0.1% saponin (Sigma).

PFA is PBS with 4% paraformaldehyde (Sigma) dissolved in it (by heating in fume hood to 65°C for 45 minutes then cooling and freezing in appropriate aliquots for use later).

2.5.2 Staining for surface markers

Cells were re-suspended to a concentration of $10^6 ml^{-1}$ in cold FACS buffer and 100μl per condition to be stained for was added to one well on a round-bottomed 96 well plate (MERCK, Germany). The plate was then spun at 300g for 5 minutes at 4°C. The supernatant was flicked off and the plate vortexed briefly to loosen the cell pellet. The cells were re-suspended in FACS buffer to 100μl per well and spun again. Having removed the supernatant and vortexed briefly, the cells were re-suspended in 25μl of FACS buffer containing the appropriate antibodies at the specified dilution (see table 2.3). This was incubated on ice in the dark for 30 minutes. After the incubation the cells were washed x3 in FACS buffer as outlined above before being re-suspended in 100μl per well of FIX buffer and transferred to

LP2 tubes (A1 Lab supplies, UK). For two layer staining instead of adding FIX buffer, the second antibody was added and the protocol was repeated.

2.5.3 Staining for intra-cellular proteins

Cells were prepared as outlined above. Before the antibody was added the cells were fixed in 50μl per well of PFA for 10 minutes at room temperature. They were then washed twice with PBS as above and then once in PERM buffer. This permeabilised the cells enabling the antibodies to enter and bind to the specific protein. After spinning and removing the supernatant the cells were re-suspended in 25μl of PERM buffer containing the appropriate antibody at the dilution outlined in table 2.3. This was then incubated on ice for 30 minutes in the dark before being washed twice more in perm buffer and once in FACS buffer, then re-suspended in 100μl FACS buffer and transferred to LP2 tubes. Again if two-layer staining was necessary instead of re-suspending in FACS at the end the cells were re-suspended in the secondary antibody and the process was repeated.

2.5.4 Antibodies used throughout the project

Most monoclonal antibodies were used as direct conjugates, conjugated to Fluorescein Isothiocyanate (FITC), R-Phycoerythrin (PE), CY (cychrome) or QR (Quantum Red). For any antibodies that were unlabelled a "second layer" conjugated reagent specific for the IgG of the relevant species was used. In some cases, no monoclonal antibody was available and polyclonal serum was used from immunised animals (rabbit, mouse) or a human patient suffering from SSPE. The antibodies used throughout the project are shown in table 2.3.

Target	Species	Clone	Conjugate	Company	Conc.
Human CD3	Mouse	-	FITC	Pharmingen	1/10
Human CD3	Mouse	UCHT-1	QR	SIGMA	1/25
Human CD3	Mouse	UCHT-1	PE	BD	1/50
Human CD4	Mouse	Q-4120	FITC	SIGMA	1/25
Human CD4	Mouse	T-310	PE	DAKO	1/40
Human CD4	Mouse	Q-1420	QR	SIGMA	1/50
Human CD8	Mouse	LEU-2A	FITC	BD Bioscience	1/25
Human CD8	Mouse	UCHT-4	PE	DAKO	1/25
Human CD8	Mouse	LEU-2A	QR	SIGMA	1/10
Human CD11a (LFA-1)	Mouse	-	FITC	Immunotech	1/25
Human CD11c	Mouse	-	FITC	Pharmingen	1/10
Human CD13	Mouse	-	PE	Pharmingen	1/25
Human CD14	Mouse	-	Cy5	SEROTECH	1/25
Human CD19	Mouse	-	PE	Pharmingen	1/25
Human CD28	Mouse	LEU-28	PE	BD	1/25
Human CD40	Mouse	-	FITC	Caltag	1/10
Human CD45RA	Mouse	F8-11-13	FITC	SEROTECH	1/25
Human CD45RA	Mouse	F8-11-13	PE	SEROTECH	1/25
Human CD45RO	Mouse	UCHL-1	PE	DAKO	1/25
Human CD80	Mouse	-	QR	Pharmingen	1/20
Human CD83	Mouse	-	FITC	Pharmingen	1/25
Human CD86	Mouse	-	FITC	SEROTECH	1/25
Human CCR7	Mouse	-	PE	R&D Systems	1/25
Human DC SIGN	Mouse	-	FITC	R&D Systems	1/50
Human DR (MHC class II)	Mouse	HK14	PE	SIGMA	1/25
Human IL-6	Mouse	-	PE	BD Bioscience	1/10
Human IL-10	Rat	-	PE	Pharmingen	1/25
Human IL-10 (blocking ab)	Mouse	23738	-	R&D Systems	10µg/ml
Human IL-12	Mouse	-	PE	Pharmingen	1/25
Human TGFß (blocking ab)	Mouse	1D11	-	R&D Systems	10µg/ml
Human TNFα	Mouse	-	PE	BD Bioscience	1/10
Human IFNγ	Mouse	-	PE	Pharmingen	1/10
Human HLA-A2*0201	Mouse	BB7.2	-	Provided by Dr Bin Gao*	1/100

Table 2.3: Antibodies used in this project

Measles	Mouse	Serum	-	LSHTM**	1/50
Measles	Human	Serum	-	LSHTM**	1/50 (1/1000 WB)
Measles	Rabbit	Serum	-	Kindly provided by Dr Kenth Gustaffson*	1/100
Measles NP	Mouse	22E1	-	Abcam	1/100
Measles NP	Mouse	-	-	LSHTM**	1/100
Measles NP	Mouse	A	-	Fabien Wilde***	1/200
Measles NP	Mouse	B	-	Fabien Wilde***	1/200
Measles NP	Mouse	C	-	Fabien Wilde***	1/200
Mouse IgG	Rabbit	-	PE	SIGMA	1/400
Mouse IgG	Rabbit	-	FITC	SIGMA	1/200
Mouse IgG	Rabbit	-	HRP	SIGMA	1/20,000 (WB)
Human IgG	Goat	-	HRP	Caltag	1/5000 (WB)
Human IgG	Goat	-	PE	Caltag	1/200
Human IgG	Goat	-	FITC	Caltag	1/200

*Table 2.3 continued: Antibodies used in this project. * Institute of Child Health, University College London (ICH, UCL). **London School of Hygiene and Tropical Medicine. ***Lyon, France.*

Where LSHTM is mentioned in table 2.3, Professor John Stephenson from the London School of Hygiene and Tropical Medicine (LSHTM) kindly donated these reagents. Where Fabien Wilde is mentioned these antibodies were kindly donated by Professor Fabien Wilde, Lyon, France. Dr Bin Gao and Dr Kenth Gustaffson are from Institute of Child Health, University College London (ICH, UCL). Where antibodies were used at different concentrations for western blot, this is denoted in table 2.3 by (WB).

2.5.5 Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE)

For analysis of phenotype of proliferating cells that responded to specific stimuli CFSE was used to stain cells and then to gate on the divided cells which had lower

levels of CFSE after division. CFSE was from SIGMA and was made to 5mM in DMSO before being aliquoted and stored at -20°C until use. To stain cells PBMC were washed to remove any serum, then counted and re-suspended in 10ml RPMI (for 10^6 to 5 x 10^7 PBMC). CFSE was added to a final concentration of 1μM and then the sample was incubated for 10mins at 37°C before 40ml of serum containing RPMI was added and the cells were washed 3 times before being re suspended at 3 x 10^6 cells per ml. 3 x 10^6 cells were added to a 24 well plate well for each condition with antigens made up to 2x in RPMI 10% FCS added 1ml per well. After the culture time, cells were harvested, washed in FACS buffer and stained with the appropriate antibodies before being analysed by flow cytometry.

2.5.6 Positive controls for proliferation assay

2.5.6.1 Phytohaemagglutinin (PHA-P)

PHA (Sigma) is a lectin extracted from the kidney bean and is a mitogen, which causes T cell proliferation. PHA was prepared in ethanol at a stock concentration of 1mgml^{-1} and was stored at -20°C. It was used at a final concentration of 1μgml^{-1}.

2.5.6.2 Tetanus Toxoid (TT)

Tetanus toxoid (TT, kindly donated by Aventis Pasteur, Marcy l 'Etoile, France) was at a stock of 2.947mgml^{-1} at +4°C and used at a concentration of 10μgml^{-1}.

2.5.7 HLA typing using flow cytometry

To HLA type different AW donors in this project for the expression of HLA-A2, the unconjugated A2 specific antibody BB7.2 (Parham and Brodsky, 1981) was used

with a second layer to stain a known positive and a known negative donor alongside the unknown donors. The cell staining protocol was followed as for surface protein staining outlined above. The antibody was kindly provided by Dr Bin Gao.

2.5.8 Data analysis for Flow cytometry

All samples were run according to standard operating procedures on a Becton Dickenson FACScan with CellQuest™ software. Live cells were gated according to their light scattering properties and typically 10,000 events were collected for each condition with the exception of proliferation assays using CFSE where 150,000 events were collected per condition. All gating was set compared to either an isotype matched control antibody or baseline staining on a comparative condition.

2.6 Monocyte derived dendritic cell (DC) culture

2.6.1 Culturing DCs

All DC work was done with RPMI media containing 5% AB human serum (Sigma) as optimisation experiments showed that this was better than FCS at maintaining healthy DC cultures. Whichever technique used to isolate the precursor population (see below), the resulting 6 day culture was performed in a 6 well tissue culture plate (NUNC) with 2ml RPMI 5% human AB serum containing 50ngml^{-1} recombinant human IL-4 (R&D Systems Inc, MN, USA) and 100ngml^{-1} recombinant human GM-CSF (R&D Systems Inc) at 37°C/5%CO_2. The cytokines arrived as lyophilised powders and were re-suspended in sterile filtered PBS 1% tissue culture grade BSA (Sigma) to a 100x concentration then stored at -70°C.

2.6.1.1 Adherence technique

After separation of PBMC from whole blood, cells were re-suspended in RPMI 5% AB human serum at a concentration of $3.3x10^6ml^{-1}$. 3ml of this cell suspension was added to each well on a 6 well plate and incubated at 37°C/5%CO_2 to adhere for 2 hours. After this incubation the non-adherent cells were removed with 3 rounds of washes with RPMI. In general these were frozen down for use as responder cells at a later date. Then 2ml of the relevant culture medium containing GMCSF and IL-4, was added per well and the cells incubated at 37°C/5% CO_2 for 6 days.

2.6.1.2 CD14 bead monocyte isolation

An alternative method of deriving DCs is to use magnetic bead separation to isolate DC precursors. This was done to compare the populations obtained by the two different methods. After isolating PBMC from whole blood cells were re-suspended in 20ml MiniMacs buffer (MMB)(2mM EDTA (from stock 0.5M pH8), 0.5% BSA in PBS, filtered and degassed for 30mins) and pelleted with a gentle spin. This was repeated and cells were re-suspended in 480µl MMB plus 1µl of anti CD-14 beads (Milteyni Biotech, Germany) per $1.5x10^6$ cells. This was then incubated at 4°C for 15 minutes with agitation every 5 minutes. 20ml MMB was then added and the cells were pelleted again at 400g for 10 minutes. Cells were then re-suspended in 2ml MMB and passed through a pre-equilibrated LT MiniMacs column (Milteyni Biotech) as per the manufacturer's instructions. Once the magnet was removed 2x 1ml of MMB was allowed to drip through the column and was collected then 3ml of MMB was plunged through and collected, the cells

were counted re-pelleted and re-suspended in the culture medium as outlined above at $0.75x10^6ml^{-1}$ and 2ml of this was seeded onto one well of a 6 well plate.

2.6.1.3 DC maturation

After 6 days in culture in GMCSF ($100ngml^{-1}$) and IL-4 ($50ngml^{-1}$) the monocyte derived DCs were ready for use. To stimulate cytokine production and phenotypic maturation, between 1 and $100ngml^{-1}$ LPS was added directly to the culture well. If looking at cytokine production using flow cytometry, Brefeldin A (Sigma), a golgi block was also added at $5\mu gml^{-1}$. Brefeldin A was reconstituted to a stock concentration of $5mgml^{-1}$ in DMSO then used at 1/1000 (final concentration of $5\mu gml^{-1}$) in the cultures for 14 hours. For work involving DCs used as stimulators the DCs were matured for 14 hours prior to use with $10ngml^{-1}$ TNFα (Sigma), $10ngml^{-1}$ IL-1β (Peprotech), $10ngml^{-1}$ IL-6 (Sigma) and $1\mu gml^{-1}$ prostaglandin E_2 (Leen et al., 2004). All cytokines were reconstituted from lyophilized powder according to the manufacturer's instruction then diluted to a 100x working stock and stored at -20°C.

2.7 Adenoviral constructs

The recombinant adenoviral constructs (Rad) used in this project are based on the replication deficient E1a and E3 genome deletion adenoviral vectors and were kindly provided by Professor John Stephenson (LSHTM). They were produced by recombination of the pJM17 plasmid (McGrory et al., 1988) with plasmids containing the cDNA sequences of the relevant proteins. The cDNA sequences for the proteins are each under the CMV promoter (see section 6.1.2). Rad68 is the

construct containing the cDNA of MV Nucleoprotein (NP), Rad88 contains the cDNA coding for MV Haemagglutinin (H) and Rad95 contains the cDNA coding for MV Fusion (F). Rad35 is the construct containing the cDNA for the reporter gene β-galactosidase which is used for all the verification of infection and effect of infection on DC phenotype and function studies. Being replication deficient the predominant proteins to be made by the host cell once the virus has infected will be the proteins of interest. As discussed further in chapter 5 this is because in the adenoviral constructs the E1 and E3 regions are deleted and this is the region of the adenoviral genome coding the transcription factors responsible for the transcription of the rest of the genome. In light of the suppressive capacity of the constructs described in chapter 6, the constructs were tested for mycoplasma and found to be negative.

2.7.1 Adenovirus amplification

All work involving growing adenovirus in 293 cells was carried out in a category 2 laboratory.

2.7.1.1 Infecting 293 cells

293 cells were grown, split the day before use and allowed to grow to 80% confluence over night in 4x T225 per viral prep. The medium was then removed and the monolayer washed gently twice with PBS. The monolayer was then covered with serum free medium and incubated at 37°C/5%CO_2 for 1 hour. Following this the medium was removed and fresh serum free medium containing 1/500 dilution of the viral stock (see below) was added. The flasks were then

incubated for a further 4 hours when this inoculum was removed and replaced with serum containing medium. These were incubated for 3 days at 37°C/5%CO_2 or until the monolayer started to float off.

2.7.1.2 Virus purification

Once the 293 cells were beginning to show the cytopathic effects of the virus, the flasks were agitated to make a cell suspension. This cell suspension was transferred to 50ml Falcon tubes and pelleted at 2000g for 5 minutes at 4°C. Each pellet was re-suspended in 2ml of cold PBS and re-pelleted. The pellets were re-suspended again in cold PBS and an equal volume of 1,1,2,trichlorotrifluoroethane (Sigma) was added and vortexed. The solution was then re-spun for 10 minutes and the aqueous layer (containing virus particles) was retained. Again an equal volume of the fluorocarbon was added and the spin repeated. The aqueous layer was then removed and divided into 300µl aliquots before being frozen for storage at -70°C. The virus preparations were kept at -70°C for at least 24 hours before titrating. To control for impurities in the preparation uninfected 293 cells were scraped off the flask and treated in the same manner to obtain a mock virus preparation which was used as a control in all the adenovirus experiments.

2.7.1.3 Plaque assays

For each viral stock to be titrated, 12 10cm diameter culture dishes were seeded with 293 cells and cultured until confluent. The medium was removed from each well and replaced with 2.5ml of serum free medium. Cells were incubated for 1 hour at 37°C/5%CO_2 then the medium removed and replaced with 0.5ml of

inoculum. The inoculum was added in duplicate at 5 different dilutions and one mock infection of medium alone. The dilutions were 1 in 10^5, 10^6, 10^7, 10^8 and10^9. The plates were then returned to the incubator for another hour and were rocked every 10 minutes. An agar overlay was prepared consisting of 50% v/v 2% noble agar (Sigma), (an agar designed to be very pure with low salt and mineral content so can be used as a gelling agent for specifically defined media) and 50% v/v 2x minimal essential medium w/o phenol red (GIBCO) 3% FCS. 3ml of this was layered gently into each well and left to set at room temperature for 15 minutes before being transferred to 37°C/5%CO_2 for 5 days. After 5 days a second overlay was prepared by the same method as the first, but containing 0.45% of 33.3% neutral red (Sigma). 3ml of this was then added to each well and allowed to set before being transferred to 37°C/5%CO_2 for a further 24 hours after this time the plaques could be counted as areas of no staining in the now red stained monolayer. The plate was left at 37°C/5%CO_2 for a further 10 days checking for the emergence of any new plaques. Routinely the most easily counted dilution was the 10^6-fold dilution and it contained approx 50 plaques. After taking into account the dilution factors, this gave a stock concentration of approx 10^8 PFU per ml (figure 5.4).

2.7.1.4 Infecting other cells

The virus preparation was added directly to cells in culture at approximately 100pfu per cell (e.g. 100μl per well of DCs).

2.7.1.5 Mycoplasma testing of virus preparation

Both mock and adenovirus preparations were tested for mycoplasma. Alongside PBMC from 3 individuals were mock or adenovirus infected and cultured for 4 days in RPMI 10% FCS without penicillin and streptomycin. The supernatants were then harvested and tested for mycoplasma. This was done using the MycoAlert Mycoplasma detection kit (Cambrex Bio Science Ltd, Belgium) according to the manufacturers instructions. Briefly the assay measures the activity of mycomplasma specific enzymes which through conversion of ADP to ADP can be measures using luciferase and measuring the luminescence of the samples, the level of ATP is measured before and after the addition of the MycoAlert substrate, resulting in a ratio value that if under 1 confirms the absence of mycoplasma. All of the samples tested were considered to be mycoplasma negative.

2.7.2 Verifying infection by adenoviral constructs

2.7.2.1 Flourescein di(B-D-glucuronide) (FDG) assay

In order to detect efficient protein expression of the protein contained in the viral DNA in Rad35 infected cells (which contain the full length β-galactosidase cDNA), a substrate of β-galactosidase (β-gal), FDG (Sigma) was used. This substrate is a colourless compound that when cleaved by β-gal becomes fluorescent and can be detected using flow cytometry in the Fl1 channel. FDG was re-suspended from lyophilised powder in a 1:1 ratio of DMSO and water to give a 200μM stock solution. This was then stored at -20°C. For working stock 10μl of this solution was added to 990μl water and incubated in a 37°C water bath for 10 minutes to dissolve. This working stock was again stored at -20°C. Cells were incubated with

Rad35 for 24, 48 or 36 hours then harvested and spun. The cells were then re-suspended in 5ml cold assay buffer (PBS 4% FCS 10mM HEPES pH 7.3) and pelleted again. The supernatant was removed and the cells re-suspended in 50μl assay buffer and incubated in a 37°C water bath for 10 minutes to recover. Then 50μl of FDG was added and the suspension put back in the water bath for 1 minute and 15 seconds (to force FDG into cells, by osmotic pressure). 1ml of ice-cold assay buffer was added and the cells left on ice for at least 3 hours before measuring using flow cytometry. While optimising this assay it was observed that cells left overnight gave a more clear difference between positive and negative cells, due to an amplification of the signal by the enzyme and this method was therefore used.

2.7.2.2 Propidium Iodide (PI)

Alongside the FDG staining of cells, 10μl Propidium Iodide (PI) (Sigma) was added to each tube to a final concentration of 25μgml^{-1} to measure cell death. Dead cells have permeable membranes therefore allow PI through the membrane. These cells can then be detected in the FI2 channel using flow cytometry. This protocol was used to investigate any association between cell infection with adenovirus and cell death.

2.7.2.3 5-Bromo-4-Chloro-3-indolyl β-D-Galactopyronoside (X-gal)

In order to directly visualize infected cells and to verify the FDG staining, the substrate X-gal was used. X-gal (Sigma) was dissolved in DMSO at a

concentration of 20mgml^{-1} this was stored at -20°C until use. This was then further diluted to1mgml^{-1} in a solution of PBS containing 5mM Potassium Ferricyanide, 5mM Potassium Ferrocyanide and 2mM $MgCl_2$ to make the working solution. Infected cells were harvested and washed twice in PBS and fixed in 4% paraformaldehyde as in section 2.5.3. The cells were then washed with PBS twice more before being resuspended in 2ml of X-gal working solution and placed in a well of a 24 well plate. The cells were then incubated for 24 hours at 37°C/5% CO_2 before viewing under a light microscope.

2.7.2.4 FACS staining for NP protein

The standard dual layer antibody labelling protocol was used to detect the NP protein by flow cytometry; this was using the Measles specific antibodies and appropriate top layers as outlined in table 2.3.

2.7.2.5 Western Blotting

Buffers and reagents.

2x SDS loading Buffer: (Tris-Cl pH 6.8, 4% w/v SDS, 0.2% w/v bromophenol blue (Sigma) and 20 % v/v glycerol)

Lysis Buffer: 1% Nonidet P-40 (Sigma) in PBS plus 10% protease inhibitor cocktail (Sigma)

Run Buffer: 0.025M Tris, 0.192M Glycine, 0.1% SDS (Flowgen)

Transfer Buffer: 0.02M Tris, 0.15M Glycine, 20% Methanol (Flowgen)

PBST: PBS, 0.05% Tween (Sigma)

Enhanced Chemiluminescence fluid (Pierce): 1ml Super signal Dura West luminal enhancer solution, 1ml stable peroxide buffer.

Protoflow gel: 30% Acrilamide, 0.8% w/v Bis acrilamide stock solution

Protoflow gel Buffer: 1.5M Tris HCL, 0.4% SDS, pH 8.8

Protoflow gel stacking Buffer: 0.5M Tris HCL, 0.4% SDS, pH 6.8

Samples were prepared as follows. Cells were re-suspended in lysis buffer (1% Nonidet P-40 (Sigma) in PBS plus 10% protease inhibitor cocktail (Sigma)) to a final concentration of 10^8 cells per ml. This was incubated for 10 minutes on ice then spun at 13,000rpm for 15 minutes in a micro centrifuge (Eppendorf centrifuge 5415R). The samples were then diluted 1 in 2 in 2x SDS loading buffer (Tris-Cl pH 6.8, 4% w/v SDS, 0.2% w/v bromophenol blue (Sigma) and 20% v/v glycerol (Sigma) and boiled for 5 minutes at 94°C. 10µl samples were added to the wells of 12% SDS protein separating gels made using the Flowgen Protogel system (Flowgen). 12% acrilamide gels were prepared by adding 6ml of protoflow gel to 3.9ml of protoflow gel buffer, 5ml of deionised water, 50µl of 10% ammonium persulphate and 10µl of NNNN tetramethyl ethylene diamine (TEMED). 4% acrilamide stacking gels were made by adding 1.3ml of protoflowgel to 2.5ml of protoflow gel stacking gel buffer, 6.1ml deionised water, 50µl 10% ammonium persulphate and 10µl TEMED. Once loaded gels were run in an Xcell SureLock mini Cell and Blot module (Invitrogen) according to the manufacturer's instructions for 1 hour at 200V or until samples had travelled sufficiently. A High Molecular Weight Rainbow Marker was run alongside the samples to allow analysis of the size of the detected protein (New England Biolabs).

The blotting of the gel was again carried out in the Xcell sureLock module at 25V for 2 hours. This was on to Hybond C Extra membrane (Amersham). The membrane was then blocked at room temperature in 5ml PBST (PBS 0.05% Tween (Sigma)) and 2.5% Marvel (Premier International Foods, UK) on a Gyro rocker (Gyro Rocker STR-9, Stuart). The membrane was washed 3 times in PBST, then the primary antibody (either rabbit, mouse or human anti measles serum at 1/1000 dilution or monoclonal anti NP at a 1/100 dilution, see table 2.3 for details) was added diluted in PBST 2.5% Marvel. This was then incubated on the rocker for 1 hour at room temperature. The membrane was washed 3 times in PBST then the secondary antibodies either anti mouse (Sigma), anti human (Caltag) or anti rabbit (Sigma)– Horse radish peroxidase (HRP) at a 1/5000 dilution in PBST 2.5% Marvel were added for 1 hour on the shaker at room temperature. The membrane was washed 5 times in PBST and then developed using Super signal west Dura luminal enhancer solution and stable peroxide buffer (Pierce). The membrane was dabbed dry with a paper towel and 2ml of enhanced chemiluminescence fluid was added evenly to the membrane. After 5 minutes the fluid was pipetted off and the membrane dabbed dry. The membrane was then wrapped in parafilm and attached to one side of an exposure box with tape. This was then used to expose Kodak X-Omat AR films (Sigma) for between 30 seconds and 2 hours.

The gels were also done in duplicate so protein content could be verified using a coomassie blue stain to ensure isolation of protein and migration of that protein through the gel. The gels were stained in 0.05% w/v coomassie blue (Sigma) solution in 50% methanol, 10% acetic acid, for 2 hours then de-stained overnight in

a 30% methanol, 10% acetic acid solution before visualization and image capture using AlphaEase software (AlphaInnotech).

2.7.2.6 Polymerase Chain Reaction (PCR)

1μl of either Rad35 or Rad68 stock virus solution was used as a template in a total reaction volume of 50μl consisting of the reagents outlined in table 2.4. The primers were designed from the known DNA sequence of the MV NP cDNA and were custom made and desalted by Sigma Genosys Ltd.

The sequences were:

NP forward: 5' ATGGCCACACTTTTAAGGAG 3'

NP reverse: 5' GTCTAGAAGATTCTGTCATT 3'

The annealing temperature used was 56°C with 30 PCR cycles on a GeneAmp PCR System 9700 (PE Applied Biosystems).

Reagent	Stock Concentration	Volume (μl)	Final concentration
PCR buffer (Tris-Cl (pH 8.4), KCl)	10X (200mM, 500mM)	5	1x (20mM, 50mM)
$MgCl_2$	50mM	1.5	1.5mM
DNTP (Invitrogen)	Each at 5mM	2	0.2mM
Primer 1	12.5μM	1	0.25μM
Primer 2	12.5μM	1	0.25μM
Taq DNA polymerase (Invitrogen)	$5U\mu l^{-1}$	0.2	1 unit
H_20		38.3	
Total		50	

Table 2.4 Reagents for PCR master mix

Negative control wells were run in parallel, omitting DNA template or primers. Gel electrophoresis of 10μl PCR product alongside 1Kb DNA ladder (New England Biolabs) was performed on 1.5% agarose Tris-borate EDTA (TBE) (1M Tris-EDTA, 0.9M Boric acid, 20mM EDTA pH 8 is a 10x solution) gel at 100V for 40 minutes. The gel was then stained in $0.5\mu g ml^{-1}$ Ethidium bromide (Sigma) in TBE and visualized by ultraviolet illumination and image capture using AlphaEase software (AlphaInnotech).

2.7.2.7 Staining for analysis by confocal microscopy

Monocyte derived DCs (see section 2.6) were harvested and counted after 6 days of culture. These were re-suspended at$10^6 ml^{-1}$ in RPMI 5% human AB serum and 1ml per well plated onto sterile cover slips (Western Lab Supplies) in 24 well plates (Corning). These were then infected with either control or test adenoviral preparations and incubated overnight at 37°C/5%CO_2. The cover slips were then removed and placed facing up in a fresh 24 well plate. These were gently washed with wash buffer (PBS 0.5% BSA) by pipetting and aspirating 1ml x4. The cover slips were then fixed in 1:1 acetone: ethanol for 15 minutes at room temperature. The fixing solution was aspirated off and the cover slips allowed to air-dry for 5 minutes. The cells were re-hydrated with 1ml of wash buffer for a further 15 minutes. The cells were then blocked on ice for 15 minutes with 200μl per well of FcR blocking reagent (Miltenyi Biotech) diluted 1/100 in wash buffer. The cells were then washed x4 as before and incubated for 30 minutes on ice with either anti measles human serum or control human serum at 1/10 dilution in wash buffer. The cells were washed x4 as above and then incubated for 30 minutes on ice in the

dark with 200μl per well of anti human IgG FITC antibody at a 1/200 dilution in wash buffer. The cells were washed x4 as before and incubated for 20 minutes on ice in 200μl of ToPro (1/500, 1μM, Molecular Probes) and phalloidin-Rhodamine (1/100 Sigma) in wash buffer. ToPro stains the nucleus by binding to DNA and the phalloidin stains the F-actin cytoskeleton. The cells were washed again x4 as before and then removed from the wells and put face down on microscope slides with a drop of fluorescent mounting medium (Dako Coporation) on them. The mount was allowed to set for 1 hour at 4°C before the edges were sealed with clear nail varnish. The slides were stored at -20°C before being studied on a Leica confocal microscope.

2.8 Statistical analysis

On the data from the vaccine study, the students paired t test was performed, comparing each time point to pre vaccination. This test was also used to test the statistical significance of the difference in responses of fresh and frozen cells. The students paired t test was also used to analyse the data from the adenovirus studies. Due to the non-parametric nature of the cytokine production data, where statistical analysis was performed a Wilcoxon paired assigned rank test was performed.

Chapter 3 Detecting measles specific responses in immune donors

3.1 Introduction

3.1.1 Detecting T cell memory

The main purpose of an immunisation protocol is to mimic the natural infection of the specific pathogen thus stimulating an immune response, which results in the appropriate memory production that is subsequently protective. The ability to study and quantify the response to both natural infection and vaccination is an essential step to better understand and develop new vaccine strategies. There are many different ways of detecting memory T cells to a specific antigen, all of which use a different parameter as a measure of memory and therefore give different information on the nature of the response.

One of the first methods to be widely used was the proliferation assay, which defined memory as the ability of cells to proliferate to a given antigen. This method used radioactive thymidine incorporation into newly synthesised DNA of proliferating cells as the read out (Mayer et al., 1996). The proliferative response can now be further defined by using carboxyfluorescein diacetate succinimidyl ester (CFSE) labelling, in which cells are labelled with a fluorescent dye, which gets divided between daughter cells on division and therefore halves in brightness, which can be detected by flow cytometric analysis (Lyons, 2000). This method has the benefit of being able to combine other staining of surface markers to phenotype the responding cells.

Another assay is the chromium release assay which measures the ability of amplified or fresh cells (typically CD8+ T cells, or NK cells) to kill antigen pulsed target cells, which have been loaded with chromium, that can be detected in supernatants, allowing killing levels to be calculated (Brunner et al., 1968).

There are several flow cytometric methods for detection of antigen specificity. One of these is tetramer staining, where a fluorescently labelled tetramer of MHC molecules loaded with a specific peptide is used to stain T cells (Appay and Rowland-Jones, 2002;Lechner et al., 2001). This often results in higher counts of antigen specific cells than other methods, on average 4.4 fold higher than levels detected by ELISpot although the two methods correlate well (Tan et al., 1999). This is likely to be because the affinity of the TCR for the tetramer may be enough to bind in the assay but not enough to promote an effector function in vivo and that the ELISpot relies on production of IFNγ as a readout which may not be the only effector function of antigen specific cells. An alternative method is intra cellular cytokine staining which detects the cells that have produced cytokine in response to the stimulus.

The benefit of flow cytometric methods is that the cell type involved in the response can be characterised with the use of surface marker staining. However these methods can be labour intensive both in laboratory work and in the data analysis, it may also require large cell numbers, therefore may not be as suitable for routine analysis as other cytokine detection methods, if only quantification of responding cells is required. The Enzyme linked immunospot assay or ELISpot is an ideal assay of this kind (Schmittel et al., 1997). The ELISpot is based on the Enzyme

linked immunosorbant assay (ELISA). However, instead of measuring levels of soluble cytokine in supernatants, the ELISpot enables quantification of the number of cells producing the cytokine. This is a direct read out of effector cells as the assay is typically 16 hours long, making this a quick and high throughput method for measuring specific cytokine responses to antigen in several cell types, not only T cells (Schmittel et al., 2001).

The ELISpot assay combined with intracellular cytokine staining were the methods used throughout this project to detect memory cells to both vaccines and natural infection. These were combined with other readouts such as proliferation assays to allow comparison with data from other groups, and CFSE staining to analyse phenotype of responding cells.

3.2 Specific Materials and Methods

3.2.1 Peptide antigens

Initially, predicted epitopes (section 3.2.1.1) were used as peptide antigens. This was subsequently followed by a more general approach, of using overlapping peptides from three of the major MV proteins as antigens.

3.2.1.1 Predicted epitopes

Table 3.1 shows the amino acid sequences of the peptides used in this project. Each peptide was allocated a code consisting of the letter representing the protein of origin and the number of the amino acid within the protein that the peptide started at. The peptides were received from Research Genetics, Huntsville, USA as lyophilised powder and were re-suspended in DMSO to a stock concentration of

10mM. For routine use these were then diluted 1/1000 (final concentration 10μM). The only exception to this was M58, which was hydrophobic and would only dissolve at 1mM so was used in assays at 1/100. Peptides were selected which were predicted to be efficiently presented by HLA-A2*0201, using a combination of reported sequences and an algorithm search (http://bimas.dert.nih.gov:80/). They were then manufactured by Research Genetics, Huntsville, USA.

Abbreviation	Origin	Protein	AA sequence
N210	Measles	Nucleo (NP)	RLERKWLDV
N226	Measles	Nucleo (NP)	DLSLRRFMV
H30	Measles	Haemagglutinin	LMIDRPYVL
C84	Measles	Core	KLWESPQEI
M211	Measles	Matrix	QLPEATFMV
M58	Influenza	Matrix	GILGFVFTL
T368	Human	Tyrosinase protein	YMDGTMSQV
G77	HIV -1	Gag	SLYNTVATL

Table 3.1 Peptides used in this study and abbreviation used in subsequent figures.

3.2.1.2 Responses to control and recall peptides

To show that antigen specific T cells could be detected efficiently in this system alongside PMA as a positive control for IFNγ release, the well characterised Flu matrix peptide M58 (Bednarek et al., 1991) was used as an antigen specific positive control, and either the HIV-1 Gag peptide or the human tyrosinase peptide which are both A2 binding peptides were used as negative controls.

3.2.1.3 Peptide pools

HPLC purified lyophilized overlapping 15mer peptides from the MV proteins nucleoprotein (NP), Fusion (F) and Haemagglutinin (H) were used. These peptides were kindly donated by Prof. Mike Steward at the LSTHM. The peptides span the majority of each MV protein and overlap each other by 5 amino acids. The sequences of the peptides are shown in tables 3.2 (NP), 3.3 (F) and 3.4 (H).

1	MATLLRSLALFKRNK	26	AEMICDIDTYIVEAG
2	FKRNKDKPPITSGSG	27	IVEAGLASFILTIKF
3	TSGSGGAIRGIKHII	28	LTIKFGIETMYPALG
4	IKHIIIVPIPGDSSI	29	YPALGLHEFAGELST
5	GDSSITTRSRLLDRL	30	GELSTLESLMNLYQQ
6	LLDRLVRLIGNPDVS	31	NLYQQMGKPAPYMVN
7	NPDVSGPKLTGALIG	32	PYMVNLENSIQNKFS
8	GALIGILSLFVESPG	33	QNKFSAGSYPLLWSY
9	VESPGQLIQRITDDP	34	LLWSYAMGVGVELEN
10	ITDDPDVSIRLLEVV	35	VELENSMGGLNFGRS
11	LLEVVQSDQSQSGLT	36	NFGRSYFDPAYFRLG
12	QSGLTFASRGTNMED	37	YFRLGQEMVRRSAGK
13	TNMEDEADQYFSHDD	38	RSAGKVSSTLASELG
14	FSHDDPISSDQSRFG	39	ASELGITAEDARLVS
15	QSRFGWFENKEISDI	40	ARLVSEIAMHTTEDL
16	EISDIEVQDPEGFNM	41	TTEDLISRAVGPRQA
17	EGFNMILGTILAQIW	42	GPRQAQVSFLQGDQS
18	LAQIWVLLAKAVTAP	43	QGDQSENELPRLGGK
19	AVTAPDTAADSELRR	44	RLGGKEDRRVKQSRG
20	SELRRWIKYTQQRRV	45	KQSRGEARESYRETG
21	QQRRVVGEFRLERKW	46	YRETGPSRASDARAA
22	LERKWLDVVRNIIAE	47	DARAAHLPTGTPLDI
23	NIIAEDLSLRRFMVA	48	TPLDIDTASESSQDP
24	RFMVALIEDIKRTPG	49	SSQDPQDSRRSAEPL
25	KRTPGNKPKIAEMIC	50	SAEPLLSCKPWQESR

Table 3.2: MV NP 15mer peptides. Each pool contained 5 peptides, when pooled pool 1 contained peptides 1,11,21,31 and 41, pool 2 contained 2,12,22,32 and 42 and so on with pool 10 containing peptides 10, 20, 30, 40 and 50.

1	IGVVGIGSASYKVMT	23	GGDLLGILESRGIKA
2	YKVMTRSSHQSLVIK	24	RGIKARITHVDTESY
3	SLVIKLMPNITLLNN	25	DTESYFIVLSIAYPT
4	TLLNNCTRVEIAEYR	26	IAYPTLSEIKGVIVH
5	IAEYRRLLRTVLEPI	27	GVIVHRLEGVSYNIG
6	VLEPIRDALNAMTQN	28	SYNIGSQEWYTTVPK
7	AMTQNIRPVQSVASS	29	TTVPKYVATQGYLIS
8	SVASSRRHKRFAGVV	30	GYLISNFDESSCTFM
9	FAGVVLAGAALGVAT	31	SCTFMPEGTVCSQNA
10	LGVATAAQITAGIAL	32	CSQNALYPMSPLLQE
11	AGIALHQSMLNSQAI	33	PLLQECLRGSTKSCA
12	NSQAIDNLRASLETT	34	TKSCARTLVSGSFGN
13	SLETTNQAIEAIRQA	35	GFSGNRFILSQGNLI
14	AIRQAGQEMILAVQG	36	QGNLIANCASILCKC
15	LAVQGVQDYINNELI	37	ILCKCYTTGTIINQD
16	NNELIPSMNQLSCDL	38	IINQDPDKILTYIAA
17	LSCDLIGQKLGLKLL	39	TYIAADHCPVVEVNG
18	GLKLLRYYTEILSLF	40	VEVNGVAIQVGSRRY
19	ILSLFGPSLRDPISA	41	GSRRYPDAVYLHRID
20	DPISAEISIQALSYA	42	LHRIDLGPPISLERL
21	ALSYALGGDINKVLE	43	SLERLDVGTNLGNAI
22	NKVLEKLGYSGGDLL	44	LGNAIAKLEDAKELL

Table 3.3: MV F 15mer peptides, when pooled pool 1 contained peptides 1-5, pool 2 contained 6-10 and so on until pool 8 containing peptides 41-44

The peptides were pooled with five peptides in each pool for the initial probing of immune responses and then any positive pools were then split to identify potential epitopes. The individual peptides were made up in DMSO at a concentration of 10mM and used at a final concentration of 10μM. The final concentration of each peptide in the pools was also 10μM. The NP peptides were pooled first in a way which separated the different overlapping residues into separate pools. However this was then deemed to be an unwise strategy as it would not allow for highlighting specific regions of the protein in specific pools, therefore the following two proteins, H and F were split into pools where consecutive peptides were included in each pool.

1	LIGLLAIAGIRLHRAAIYTAEIH	29	DPVIDRLYLSSHRGV
2	SLSTNLDVTNSIEHQ	30	SHRGVIADNQAKWAV
3	SIEHQVKDVLTPLFK	31	AKWAVPTTRTDDKLR
4	TPLFKIGGDEVGLRT	32	DDKLRMETCFQQACK
5	VGLRTPQRFTDLVKF	33	QQACKGKIQALCENP
6	DLVKFISDKIKFLNP	34	LCENPEWAPLKDNRI
7	KFLNPDREYDFRDLT	35	KDNRIPSYGVLSVDL
8	FRDLTWCINPPERIK	36	LSVDLSLTVELKIKI
9	PERIKLDYDQYCADV	37	LKIKIASGFGPLITH
10	YCADVAAEELMNALV	38	PLITHGSGMDLYKSN
11	MNALVNSTLLETRTT	39	LYKSNHNNVYWLTIP
12	ETRTTNQFLAVSKGN	40	WLTIPPMKNLALGVI
13	VSKGNCSGPTTIRGQ	41	ALGVINTLEWIPRFK
14	TIRGQFSNMSLSLLD	42	IPRFKVSPYLFNVPI
15	LSLLDLYLGRGYNVS	43	FNVPIKEAGEDCHAP
16	GYNVSSIVTMTSQGM	44	DCHAPTYLPAEVDGD
17	TSQGMYGGTYLVEKP	45	EVDGDVKLSSNLVIL
18	LVEKPNLSSKRSELS	46	NLVILPGQDLQYVLA
19	RSELSQLSMYRVFEV	47	QYVLATYDTSRVEHA
20	RVFEVGVIRNPGLGA	48	RVEHAVVYYVYSPSR
21	PGLGAPVFHMTNYLE	49	YSPSSFSYFYPFRL
22	TNYLEQPVSNELSNC	50	YPFRLPIKGVPIELQ
23	ELSNCMVALGELKLA	51	PIELQVECFTWDQKL
24	ELKLAALCHGEDSIT	52	WDQKLWCRHFCVLAD
25	EDSITIPYQGSGKGV	53	CVLADSESGGHITHS
26	SGKGVSFQLVKLGVW	54	HITHSGMEGMGVSCT
27	KLGVWKSPTDMQSWV	55	GVSCTVTREDGTNRR
28	MQSWVPLSTDDPVID		

Table 3.4: MV H 15mer peptides. When pooled pool 1 contains peptides 1-5, pool 2 6-10 and so on until pool 11 containing peptides 51-55

3.2.2 Determining MV immunity

To determine if volunteers were immune to MV, as defined by serum antibody concentration, serum samples were prepared from each donor and kindly analysed by Dr Bernard Cohen at the Enteric, Respiratory and Neurological Virus Laboratory at the Health Protection Agency (HPA), London, for titres of anti MV antibody. Quantitative measurements of anti measles IgG were made using an ELISA kit

made by the Dade Behring company which uses plate bound MV antigen and a standard ELISA method to measure anti MV IgG. This is compared to WHO standard serum to give ab titres in international units.

3.3 Results

3.3.1 Optimisation of the ELISpot.

The IFNγ ELISpot protocol outlined in the materials and methods (chapter 2) was optimised as outlined in the experiments below. In all ELISpot data, data points represent the mean of triplicate wells with error bars showing the standard deviation (SD). The data are shown either alongside the control value (the number of spots produced in a well with no antigen or in later experiments using the tyrosinase control peptide, a self antigen which should not be recognized, or the HIV-1 peptide in volunteers assumed to be HIV-1 negative) for comparison, or in vaccine studies with the background subtracted to allow easier comparison of different time points. Each plate included a positive control well of PMA (data not shown). Typically positive control wells showed in excess of 250 spots per 250,000 PBMC.

3.3.1.1 Optimising cell number

To ensure the optimal number of cells was being used per well, taking both low precursor frequency of reactive cells and background into consideration, titrations of cell number were carried out (figure 3.1). This showed that over a certain cell density, cells began to non-specifically release IFNγ. Thus 250,000 cells per well were used in experiments using specific antigen, unless otherwise stated. In

optimisation experiments using PMA the response was high enough to use only 100,000 cells per well as in figures 3.2 and 3.3.

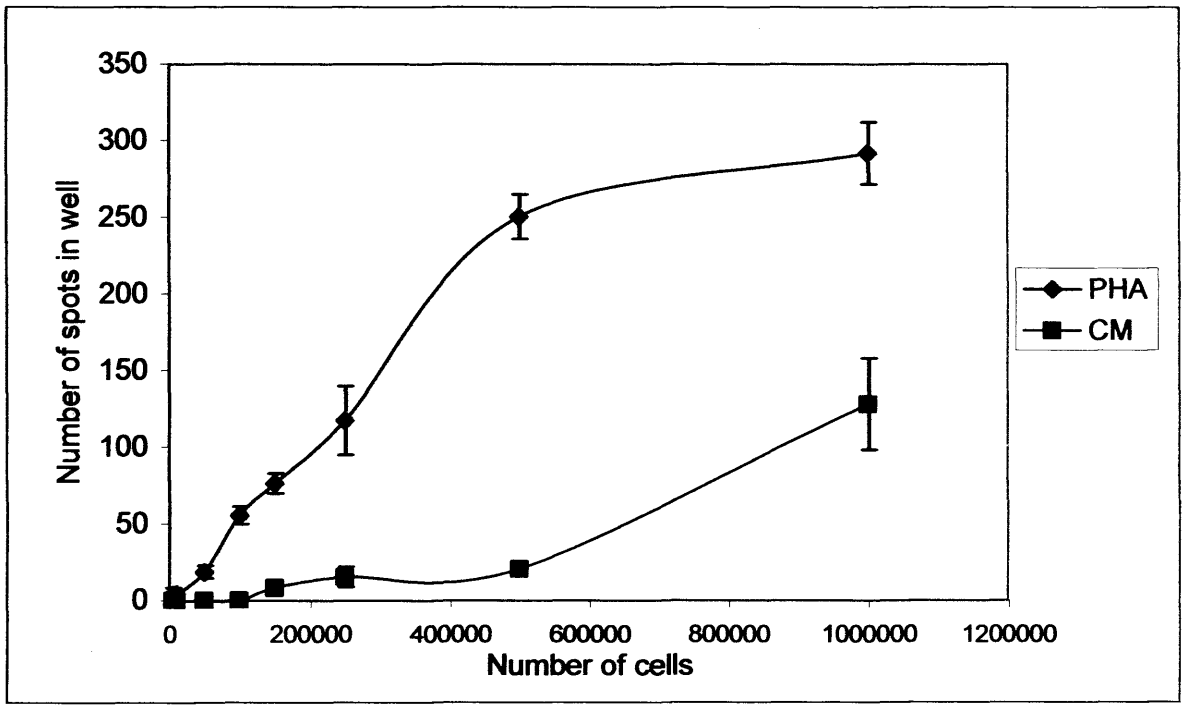


Figure 3.1 Titration of cell number per ELISpot well. Graph showing the number of spots produced by different numbers of PBMC from one donor, either un-stimulated (pink) or stimulated (blue) with 1μgml^{-1} PMA in a standard ELISpot. Data points represent mean spots in 3 wells with error bars representing the SD.

3.3.1.2 Primary antibody titration

The optimal concentration of primary antibody was first determined. Figure 3.2 shows how the detection level falls off rapidly with increasing dilution. Thus, the primary antibody was used at a 1.5% of stock solution, which was equivalent to a 1/66.6 dilution, a final concentration of 15μgml^{-1}.

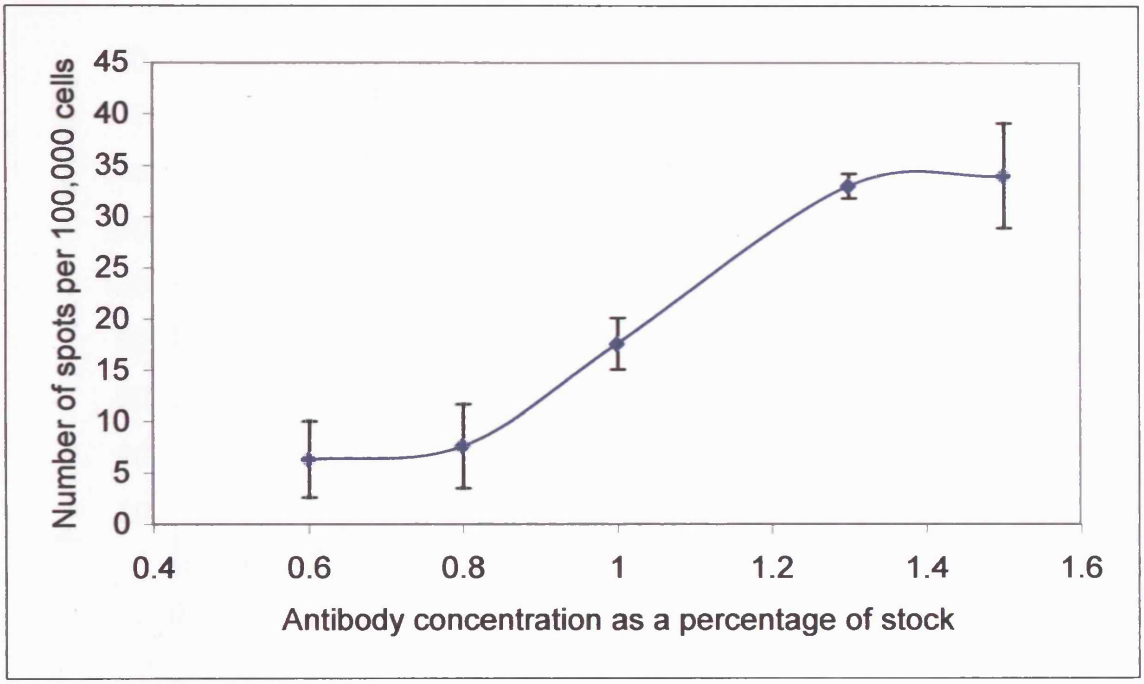


Figure 3.2 Titration of primary antibody concentration. 100,000 PBMC from one donor were incubated with 1μgml^{-1} PMA in a standard ELISpot except with increasing dilutions of the primary antibody from 0.75 to 1.5% of the stock solution. Data points represent mean spots in 3 wells with error bars representing the SD.

3.3.1.3 Titration of PMA

As this assay was a new protocol in the laboratory, experiments were carried out to ensure the assay could detect an increasing number of responder cells in a linear fashion in this setting. A titration of PMA was carried out the results of which are outlined in figure 3.3. This titration was performed using 100,000 cells per well using concentrations of PMA ranging from 0.1 to 1ngml^{-1}. This experiment shows the assay to be both sensitive and reproducable over a wide range of number of responding cells.

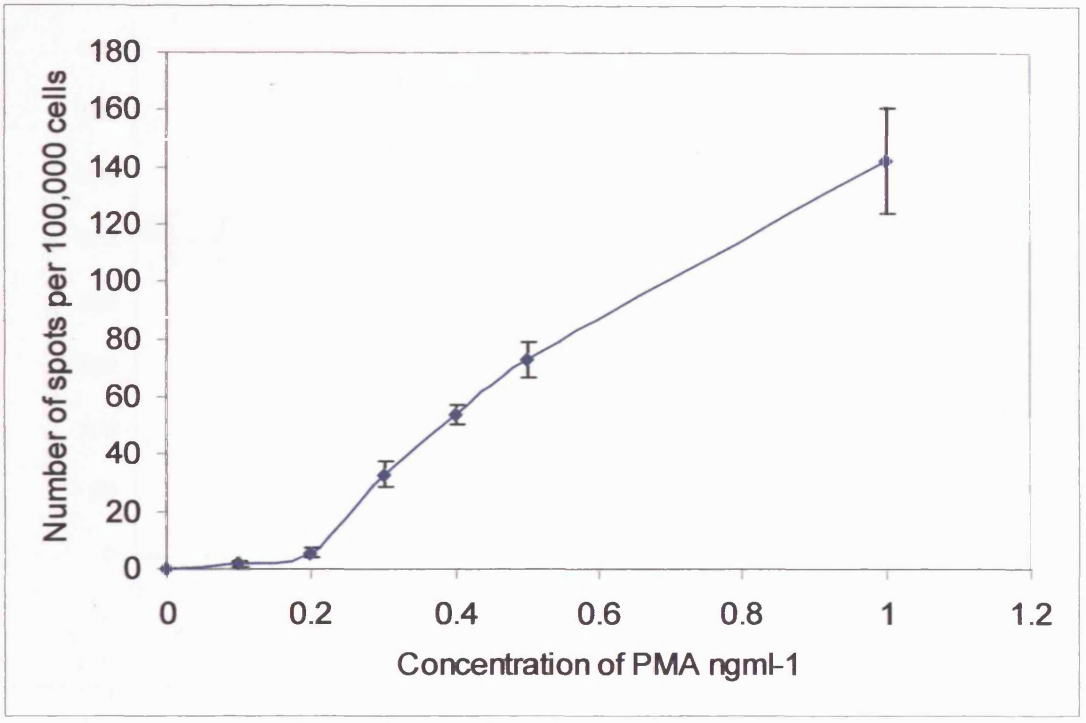


Figure 3.3 Titration of PMA in the ELISpot. 100,000 PBMC from one donor were incubated in a standard ELISpot with increasing concentrations of PMA, from 0 to 1ngml^{-1}. Data points represent mean spots in 3 wells with error bars representing the SD.

3.3.1.4 Titration of DMSO

There was concern that DMSO (which was used as a diluent for peptide preparations) may have a toxic effect on PBMC. Therefore a titration (figure 3.4) was performed to assess any such effect. This measured spots formed in response to PMA with different concentrations of DMSO. The final concentration of DMSO in the cultures after addition of peptide was 0.1% (with the exception of 1% for M58 and 0.5% for the peptide pools). Due to scale 5% DMSO was not included on the graph. However at this concentration of DMSO there was a marked decrease in spots formed (data not shown).

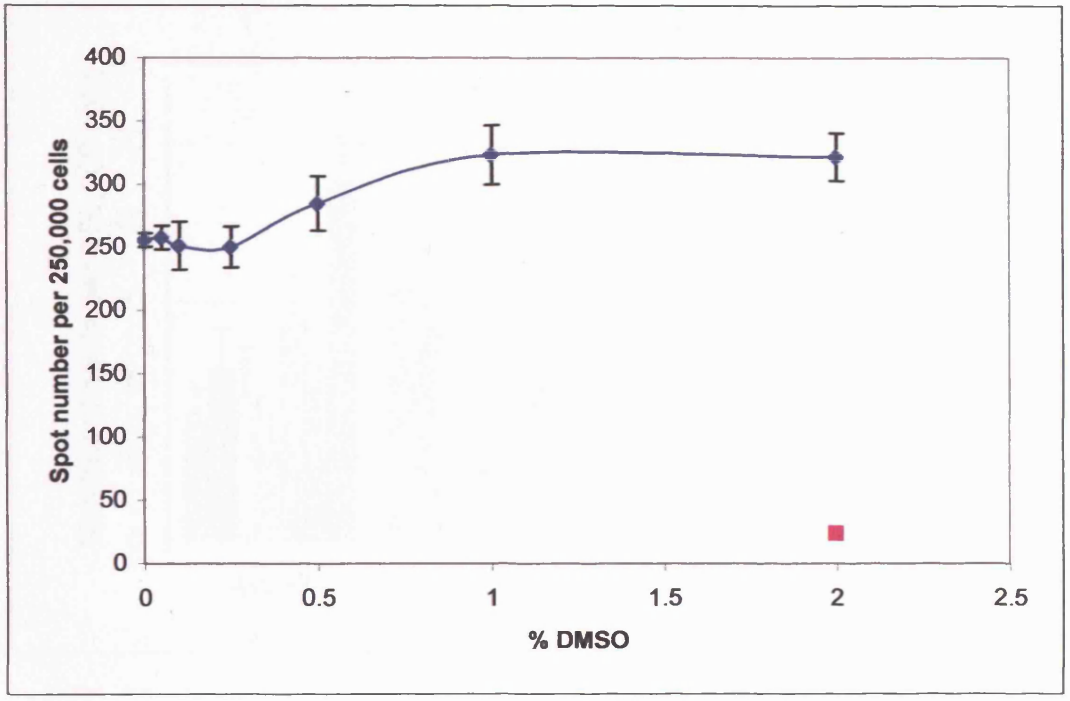


Figure 3.4: DMSO titration in the ELISpot. 100,000 PBMC from one donor were incubated with 1μgml^{-1} PMA and an increasing concentration of DMSO (blue). Shown in pink is the number of spots made with no PMA in the presence of 2% DMSO. Levels of 5% DMSO did have a detrimental effect of the number of spots (data not shown). Data points represent mean spots in 3 wells with error bars representing the SD.

3.3.2 Preliminary ELISpot results

Once the ELISpot method had been optimised, the measles peptides and positive controls were assessed. All individuals used in the initial ELISpot experiments were shown to be HLA-A2*0201 positive by genotyping, and immune to MV as defined by positive serology. The results from 4 adult well (AW) volunteer ELISpots are shown in figure 3.5. Very little specific IFNγ production could be detected to any of the peptides except the positive control M58 from influenza matrix protein in 3 of the 4 individuals, known to be recognised in the context of HLA-A2*0201 (Bednarek et al., 1991).

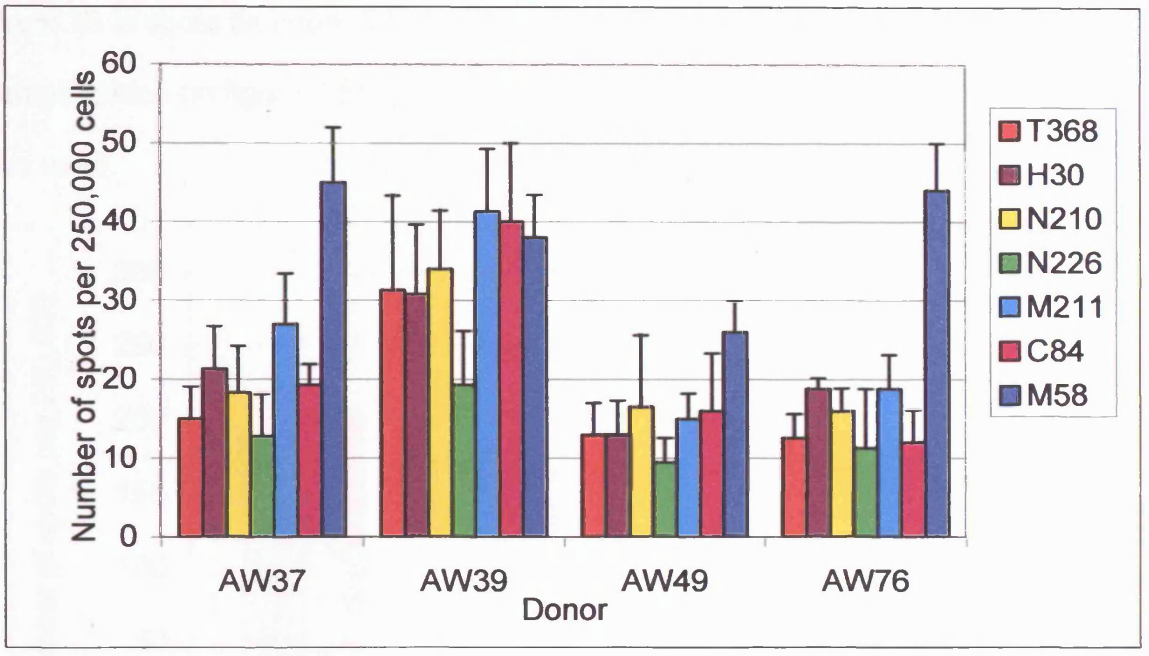


Figure 3.5: Preliminary measles peptide results. A standard ELISpot was carried out with 250,000 PBMC per well, stimulated with different peptides at a concentration of 10μM. Comparing measles peptide responses with the negative control peptide T368 and the positive control M58 in 4 adult well (AW) donors. Bars represent mean spots in 3 wells with error bars representing the SD.

3.3.3 Long ELISpot

Given the lack of specific responses seen in response to predicted A2 epitopes derived from MV, possible explanations for these negative results were sought. It was possible that the response to peptides would require a longer incubation time, in order to make equivalent levels of IFNγ as that made in response to PMA. Therefore to test whether this might be the reason for the lack of response seen to peptides (figure 3.5), a 48-hour ELISpot was performed (in which the incubation time of PBMC with peptide antigen was 48hr) instead of the usual 16 hour ELISpot. The results of this assay carried out in 4 healthy donors using 4 different peptides or DMSO alone are shown in figure 3.6. The only effect this had was an increase in background as shown in the control well with an increase in AW49 background

from 15 in spots (in figure 3.5) to 125 spots and no detectable specific response amplification (in figure 3.6). Therefore the standard 16-hour incubation continued to be used.

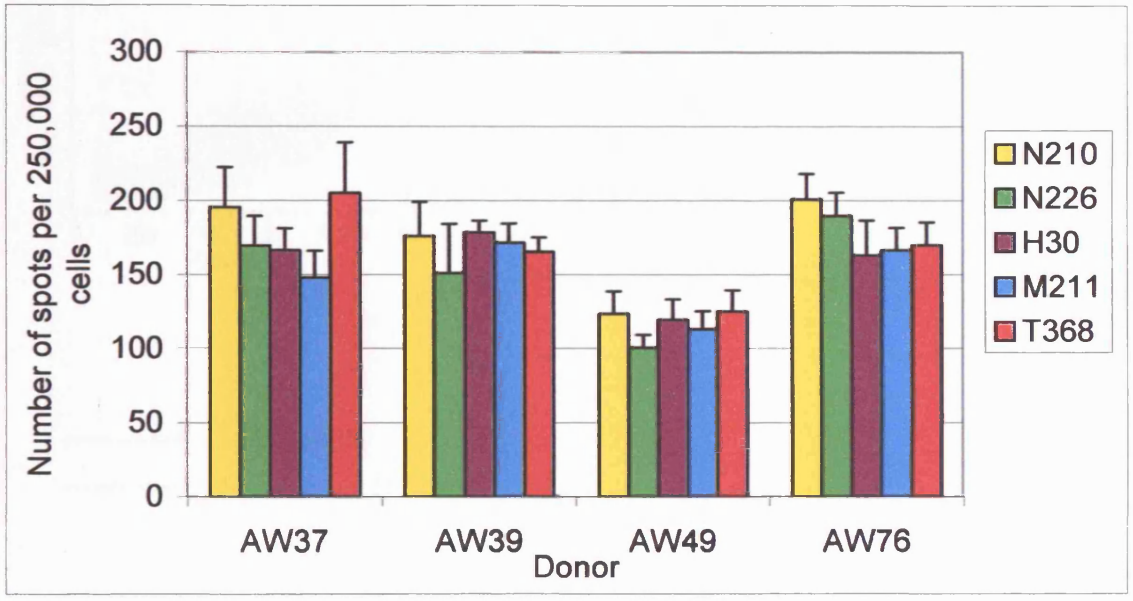


Figure 3.6: Long ELISpot results. A standard ELISpot was performed with 250,000 cells per well incubated for 48hrs rather than the usual 16hrs with 10μM of peptide antigen in the same 4 healthy donors as shown previously in a 16hr ELISpot. Bars represent mean spots in 3 wells with error bars representing the SD.

3.3.4 Using Antigen Presenting Cells (APC) to enhance responses.

Due to the lack of response seen in figure 3.5, the issue of efficient antigen presentation was raised. It was possible that low frequency of professional antigen presenting cells (APC) could limit the detectable responses, even though memory CTL frequencies were under investigation. Therefore several different cell types were tested in the ELISpot as APC, as described in 3.3.4.1-3.3.4.3.

3.3.4.1 Autologous dendritic cells (DC)

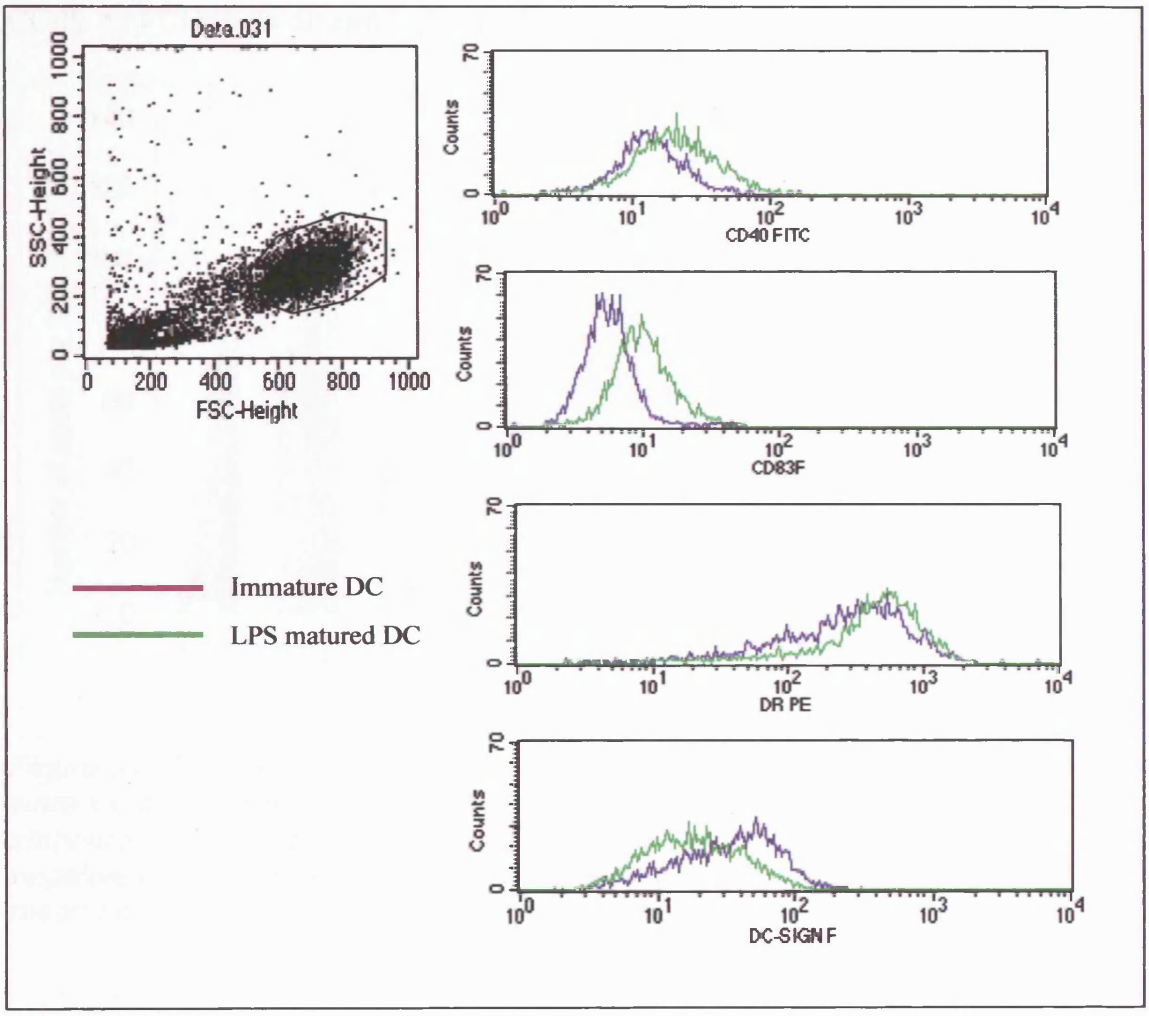


Figure 3.7: Human monocyte derived DCs. Scatter plot (top left) and phenotype histograms of flow cytometric analysis of monocyte derived DCs pre (purple line) and post (green line) LPS induced maturation. The results show up regulation of CD83, CD40 and HLA-DR, and down regulation of DC-SIGN on the surface of myeloid DCs, consistent with maturation of the DC (Banchereau and Steinman, 1998b;Renneson et al., 2005). DCs were analysed before or after maturation with LPS for the surface markers as shown and antibody stained cells run on the flow cytometer. Histograms show cells gated as shown in the dot plot.

Monocyte derived DCs were generated by standard adherence to plastic method followed by 6 day culture in GMCSF and IL-4 (see section 2.6.1). This generated a homogeneous population of cells with high expression of DC-SIGN and MHC Class II (figure 3.7) and low expression of CD14 (data not shown). These cells, on

receiving a maturation signal such as LPS, also up regulated markers such as CD40 and CD83 as shown in figure 3.7.

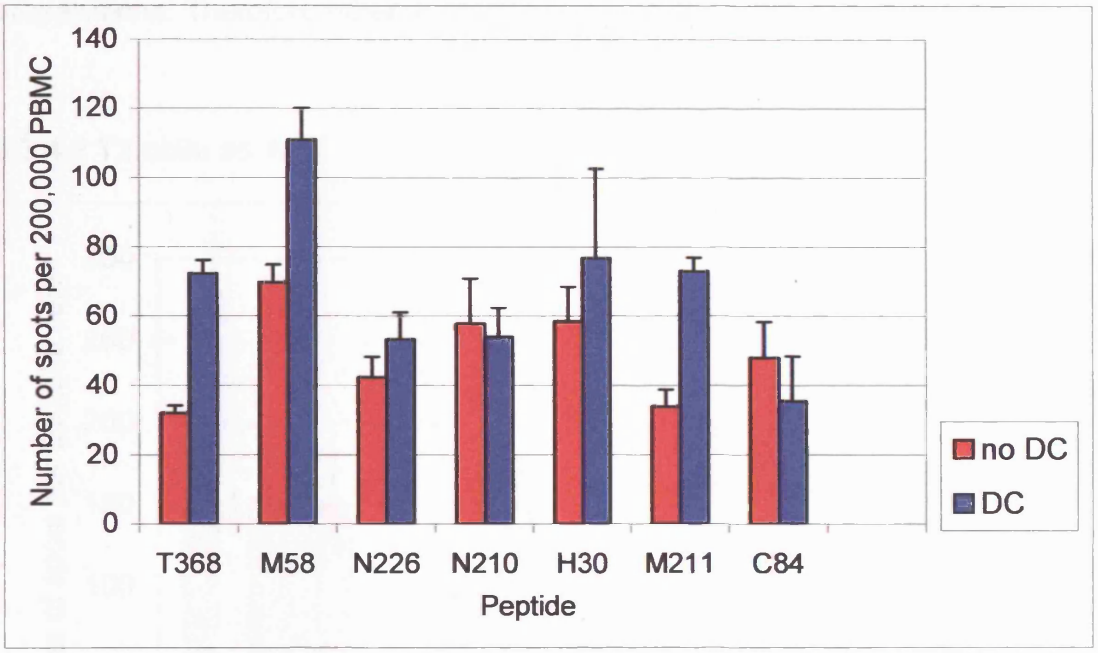


Figure 3.8: Responses to peptide presented on autologous DCs. 200,000 PBMC were incubated with 50,000 DCs after peptide loading and without peptide in a standard ELISpot assay. T368 is used as a self protein derived (tyrosinase), negative control peptide and M58 as a positive control peptide. Bars represent mean spots in 3 wells with error bars representing the SD.

Monocyte derived DCs were pre-incubated with peptide for 3 hours then washed and used at a 1:4, DC: PBMC ratio as described in section 2.4.1. This was with 200,000 cells per well from the non-adherent (containing T cells) fraction of PBMC which had been stored after the DC preparation. These numbers were chosen in order not to exceed the 250,000 cells per well limit (section 3.3.1.1). The results of this, from one donor (representative of 2 experiments) are shown in figure 3.8. While this method appeared to improve antigen specific responses detected to M58 and in the case of one MV peptide M211, it also increased background responses to the irrelevant tyrosinase peptide, T368. Also, the limitations imposed

upon experiment size due to using blood samples from children (planned for the future) would mean that there may not be enough cells to carry out standard DC preparations. Therefore other methods of presenting antigen were investigated.

3.3.4.2 T2 cells as APC

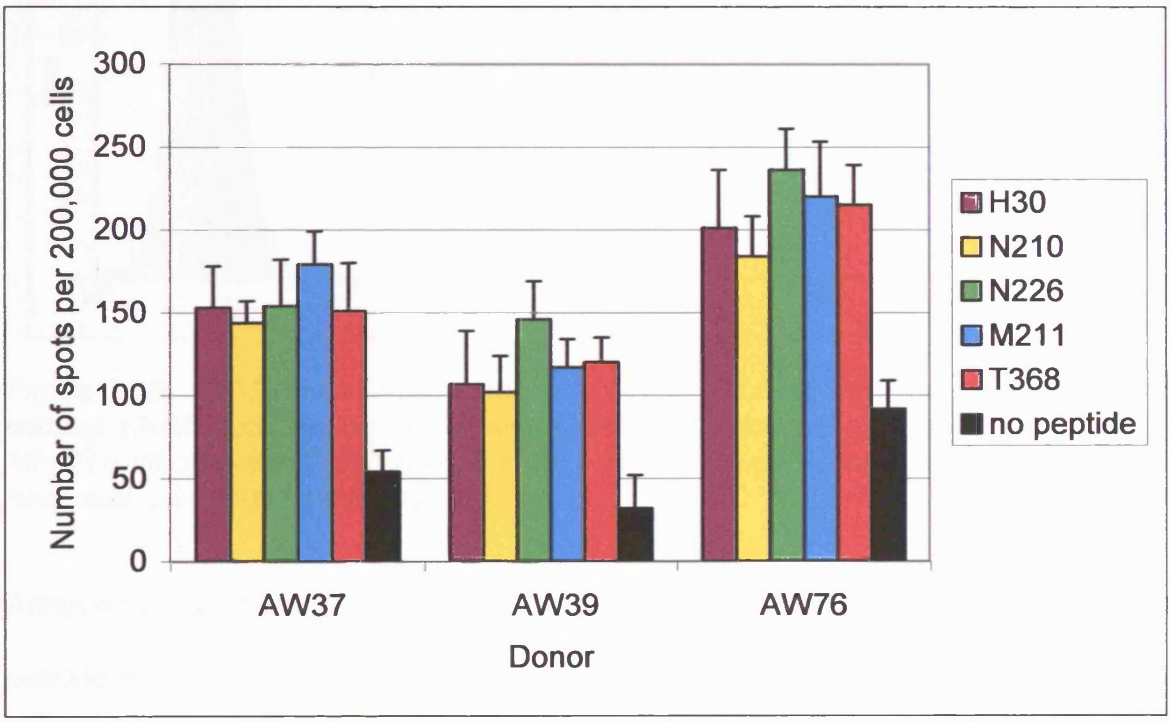


Figure 3.9: Responses (mean of 3 wells +/- SD) to peptide presented on T2 cells.200,000 PBMC were incubated with 50,000 T2 cells after peptide loading and without peptide in a standard ELISpot assay. T368 is used as a negative control peptide.

The T2 cell line is a cell line deficient in TAP the transporter gene for antigenic peptide (Zweerink et al., 1993). Therefore these cells are unable to process antigen and as such should have no MHC Class I on the cell surface as this molecule is unstable when no peptide is bound (Zweerink et al., 1993). T2 cells were pre-incubated for 3 hours with the different peptides at a concentration of 10μM, washed and then 50,000 peptide loaded T2 cells were added per well with

200,000 PBMC in a standard ELISpot assay. The results of this approach for 3 donors are shown in figure 3.9.

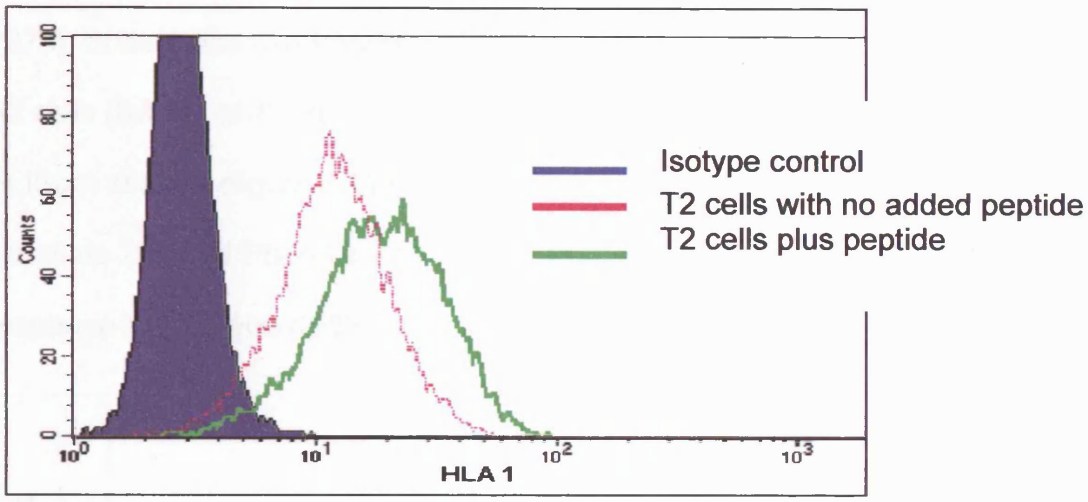


Figure 3.10: MHC Class I expression on T2 cells. T2 cells were incubated with and without 10μM M58 peptide for 3 hours before being washed and stained with anti MHC I antibody W6-32 and an anti mouse FITC conjugated second layer then analysed using flow cytometry

Although the background response to T2 was low, the response when the control peptide was added increased as much as to the measles peptides, indicating that the PBMC were responding to other signals. Therefore T2 cells were analysed using flow cytometry (figure 3.10) to compare levels of MHC Class I expression both after incubation with 10μM M58 peptide and without. Although incubation in M58 did increase levels of MHC Class I, the T2 cells were seen to have a high level of MHC class I expression before exogenous peptide was added (figure 3.10). Therefore, as the T2 cells did not facilitate the demonstration of MV specific T cell responses, this line of investigation was not pursued any further.

3.3.4.3 K562 cells

The K562 cell line is a human lymphoma cell line that is usually used as an NK cell target due to the lack of MHC Class I expression on its surface (Andersson et al., 1979). Britten et al had transfected this cell line with HLA-A2*0201 to make K562-A2 cells (Britten et al., 2002). This new cell line was generously donated for use in ELISpot assays. Figure 3.11 shows a titration of wild type K562 cells used to stimulate 200,000 PBMC in an ELISpot, in 4 donors which shows that there is a response to wild type K562 cells.

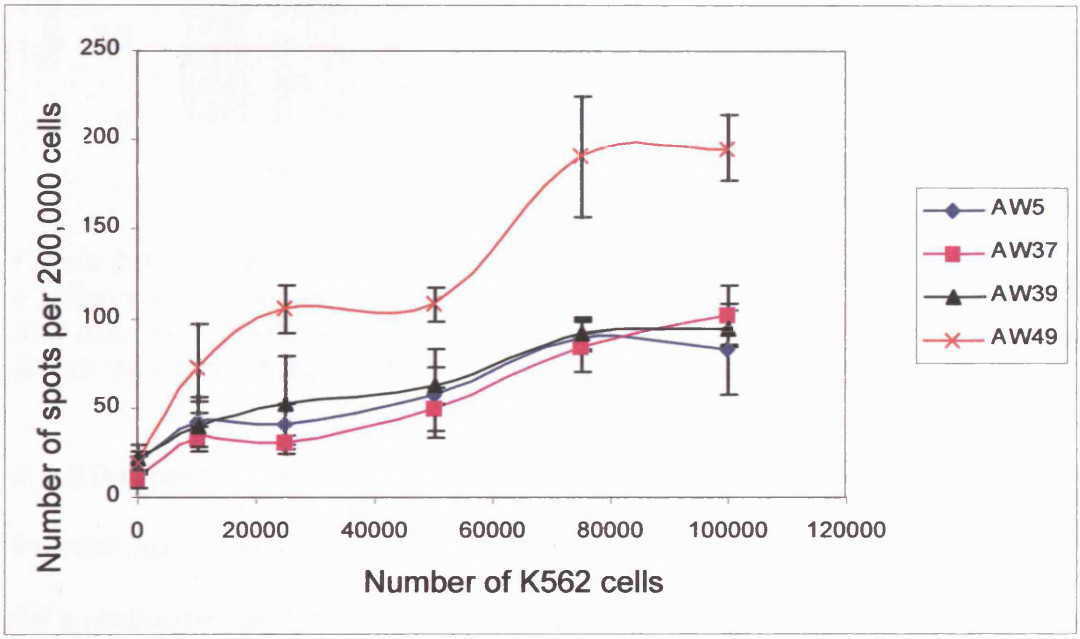


Figure 3.11: K562 cell titration. Standard ELISpots were carried out using 200,000 PBMC per well with increasing numbers of wild type K562 wild type cell line, from 0 to 100,000 cells per well. This was done in 4 donors. Data points represent mean of 3 wells +/- 1SD.

Figure 3.12 shows the K562-A2 cells that had been pre incubated for 3 hours with 7 different peptides at a final concentration of 10μM (5 MV derived peptides, the negative control tyrosinase peptide and the positive control flu matrix peptide). Due to the large background, the response to M58 the positive control became much

less obvious in all of the donors tested. As with the T2 cells, the background response to K562 cells was found to be very high and so this cell line too was no longer used.

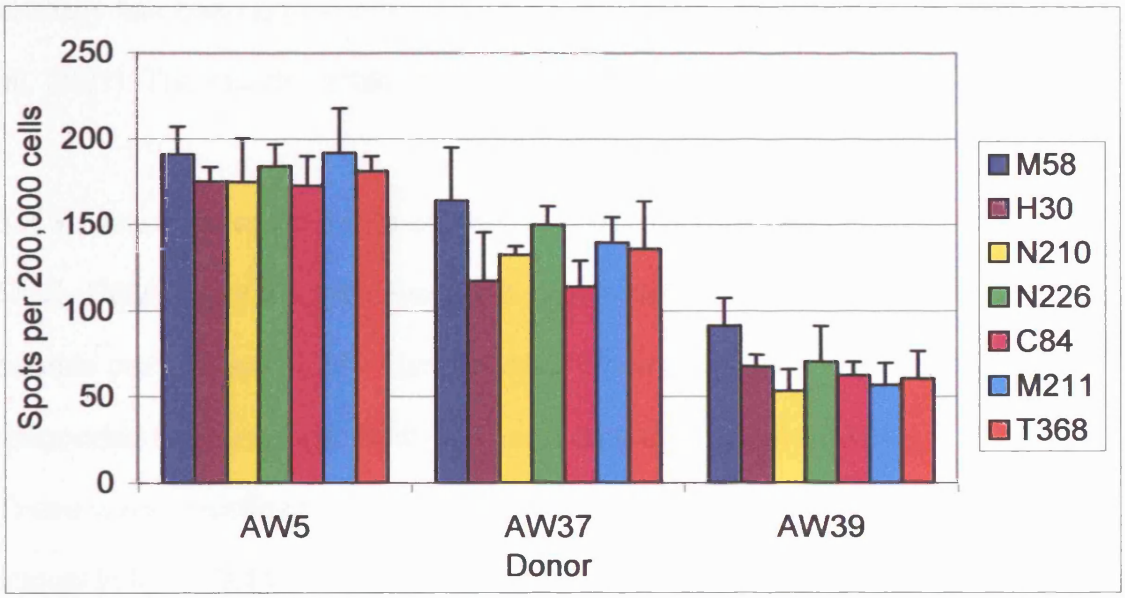


Figure 3.12: Responses to K562-A2 cell lines (mean of 3 well +/- 1SD). Standard ELISpots were carried out with 200,000 PBMC per well and 50,000 K562-A2 cells that had been incubated with 10μM peptide and washed. This was done for the 5 MV derived peptides and the positive and the negative control peptides.

3.3.5 Responses to MV Peptide pools in normal MV immune control individuals

As a response had not been observed to the predicted peptides and efforts to enhance presentation of these peptides had not increased the response to the peptides over background responses, it was decided to use a different approach. Prof Mike Steward at the LSHTM kindly donated peptides that had been generated according to the sequences of 3 MV proteins, NP, H and F. These peptides were all 15 amino acids long and overlapped by 5 amino acids (table 3.2, 3.3 and 3.4). The peptides were pooled, with 5 peptides in each pool, giving 10 NP pools, 9 F pools and 11 H pools which could be used to probe the responses to the three

proteins in immune individuals. Pools of peptides were used as antigen in standard ELISpot assays to stimulate PBMC for MV immune donors. This strategy uses the natural APC within the PBMC to present antigen to the T cells in the assay. This strategy has been previously used to successfully identify HIV epitopes(Goulder et al., 2001). The results of this strategy are shown in figures 3.13-3.15.

3.3.5.1 Responses to pools of MV Nucleoprotein peptides

Firstly PBMC from 4 donors were tested in a standard ELISpot with the 10 NP peptide pools (figure 3.13). This identified three pools that appeared to be responded to more consistently between donors, these were pools 2, 6 and 10. These were investigated further by splitting the pools in two of the donors this is shown in figure 3.14. Pool 4 was also spit to ensure the low responses to this pool were also reproducible in the split pool assay.

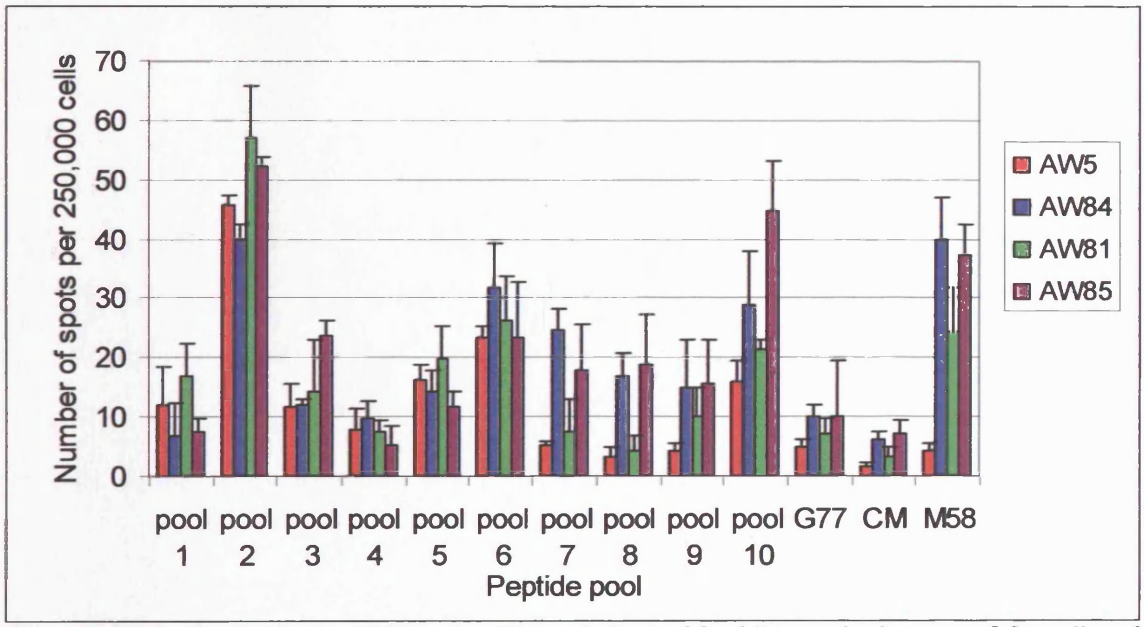


Figure 3.13: Responses of four healthy adults to MV NP pools (mean of 3 wells +/- 1SD). Standard ELISpots were carried out stimulating 250,000 PBMC per well from 4 MV immune donors with 10 pools of peptides derived from the MV NP protein, HIV negative control peptide and M58 positive control peptide at 10μM final concentration.

In analysis of responses to peptides from MV NP, the pool that most individuals had consistently responded to was pool 6 and pool 2 (figure 3.13). However on splitting pool 2 no significant response was seen in more than 1 donor for more than 1 peptide. There was a peptide within pool 6 that all individuals responded to and this was peptide 6 (figure 3.14 and data not shown). The sequence of this peptide was LLDRLVRLIGNPDVS which interestingly on analysis contained a sequence that had similarity to the A2- binding motif of LXXXVXXVX, in this case LDRLVRLIG, the final valine being replaced with an isoleucine. However valine (V) and isoleucine (I) are similar in property and structure so it is possible that this peptide could bind to HLA-A2. The exact peptide epitope 9mer was manufactured by Peprotech and referred to as N77. Another 9mer was made that was frame shifted to the right so the sequence was DRLVRLIGN and was referred to as N78. The original peptide 6 15mer was also re-made as the initial stock was running low.

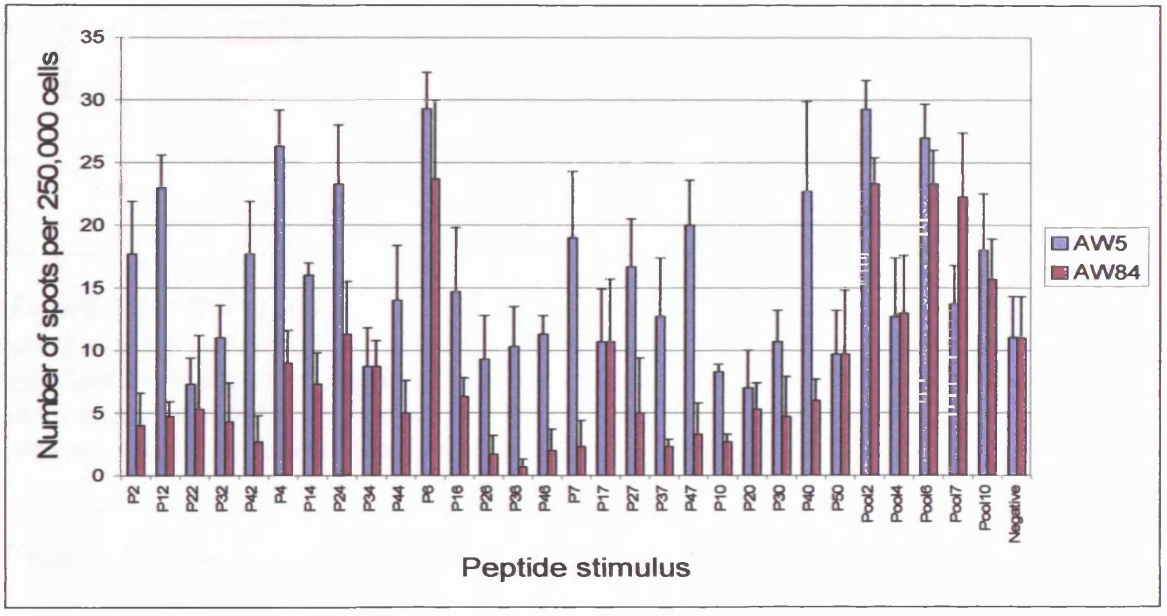


Figure 3.14: Responses to split MV NP pools 2, 4, 6, 7 and 10 in 2 healthy adults (mean of 3 wells +/-1SD). Standard ELISpots were carried out in 2 donors splitting pools of peptides that had been responded to in previous experiments. Peptides were used at 10μM to stimulate 250,000 PBMC per well.

When the new peptides were investigated in the ELISpot alongside the original peptides it was found that only the original peptide 6 produced a response (figure 3.15) and neither of the 9mers produced a response either (figure 3.16). This suggested that the original peptide 6 may have been contaminated and that it was not a true antigen specific stimulation that was being observed. This hypothesis was strengthened by the observation that 4 non A2 donors also responded strongly to the original peptide 6 but not the new peptide 6 (data not shown).

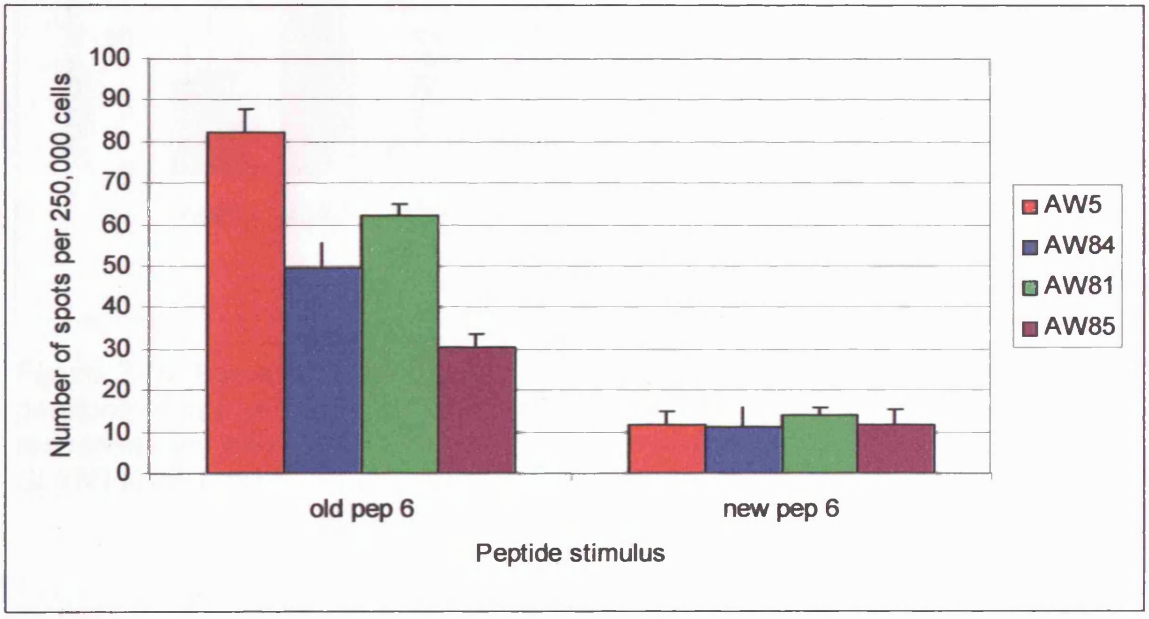


Figure 3.15: Comparison of responses to original and new peptide 6 in 4 healthy adult donors indicating the original peptide may have been contaminated. Standard ELISpots were carried out to compare responses of 250,000 PBMC per well from 4 donors to the original peptide 6 15mer with the newly synthesized 15mer at 10μM. Values shown are mean spots in 3 wells, error bars represent one SD.

Thus, after initial data generated by dividing the NP peptide pools in healthy adults was interesting the final conclusion of this section of the work was that it did not highlight any one peptide as being dominant in several individuals tested.

Therefore this approach did not allow confident identification of HLA-A2*0201 restricted peptides from the MV NP protein.

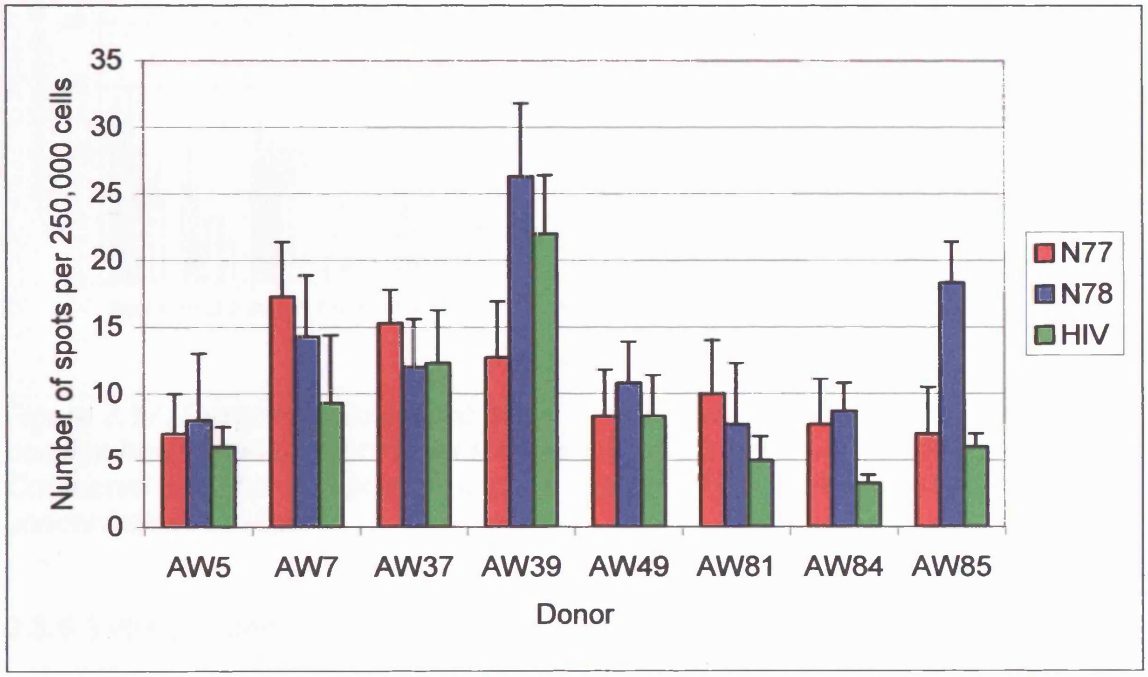


Figure 3.16: Response of 8 HLA-A2 positive individuals to the N77 and N78 MV peptides (mean of 3 wells+ /-SD) in a standard ELISpot, showing no increase in response over background responses to HIV negative control peptide (Gag SLYNTVATL).

3.3.5.2 Responses to MV Haemagglutinin (H) protein peptide pools

As shown in figure 3.17 there was no significant increase in response in any of the peptide pools from MV H protein in any of the individuals tested (n=4) over background responses. Therefore it was decided not to split any of the pools or investigate these peptides any further in normal un- boosted individuals.

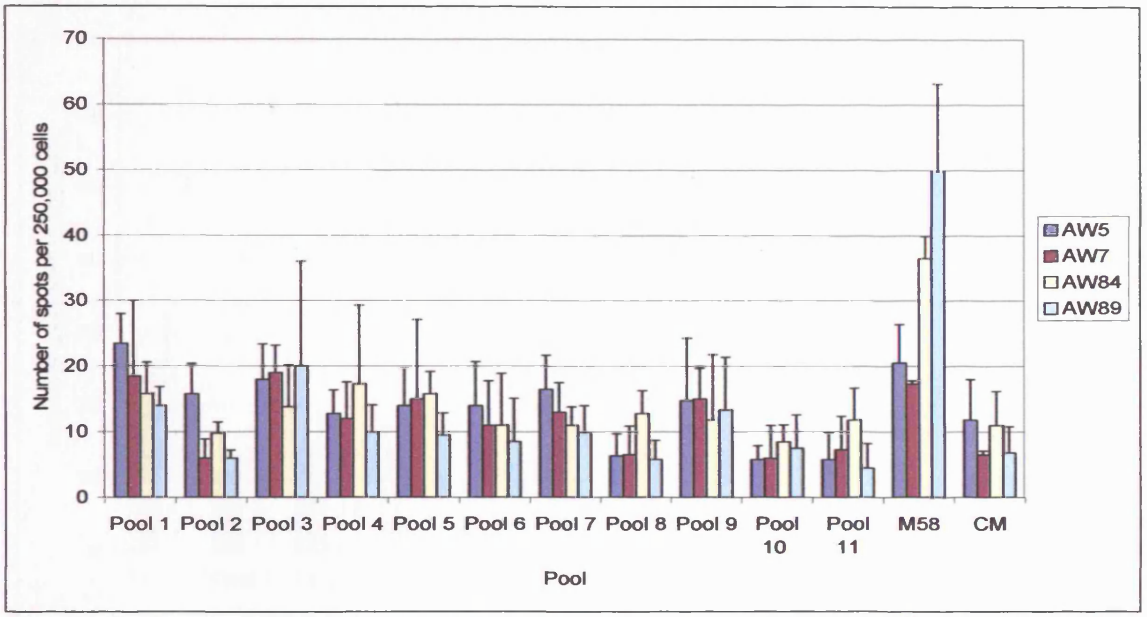


Figure 3.17: Responses to pooled MV H protein peptides of 4 MV immune HLA-A2 positive healthy adult donors in a standard ELISpot (mean of 3 wells +/-1SD). Compared with the M58 positive control peptide all peptides were used at a concentration of 10μM.

3.3.5.3 Responses to Fusion (F) protein peptide pools

Figure 3.18 shows the responses in four normal MV immune HLA-A2 positive individuals to the MV F protein peptide pools. It shows that peptide pools 6, 7, 8, and 9 might be interesting to divide and look at individual peptide responses. Therefore MV F protein pools 6, 7, 8 and 9 were split and individual peptides used to stimulate PBMC of the same 4 normal volunteers. This is shown in figure 3.19. The most obvious response to see in figure 3.19 is that to peptide 34 which has the sequence TKSCARTLVSGSFGN which does not contain any obvious HLA-A*0201 binding motif sequences. On further investigation of the healthy adult volunteers all were found to also have C*0602 or 0702 in common. The binding motif for these alleles is still unknown. Peptide 27 which has the sequence GVIVHRLEGVSYNIG (figure 3.19) was also recognised. There is also no obvious A2 sequence in this peptide and no other peptides that induced a response in the individuals tested.

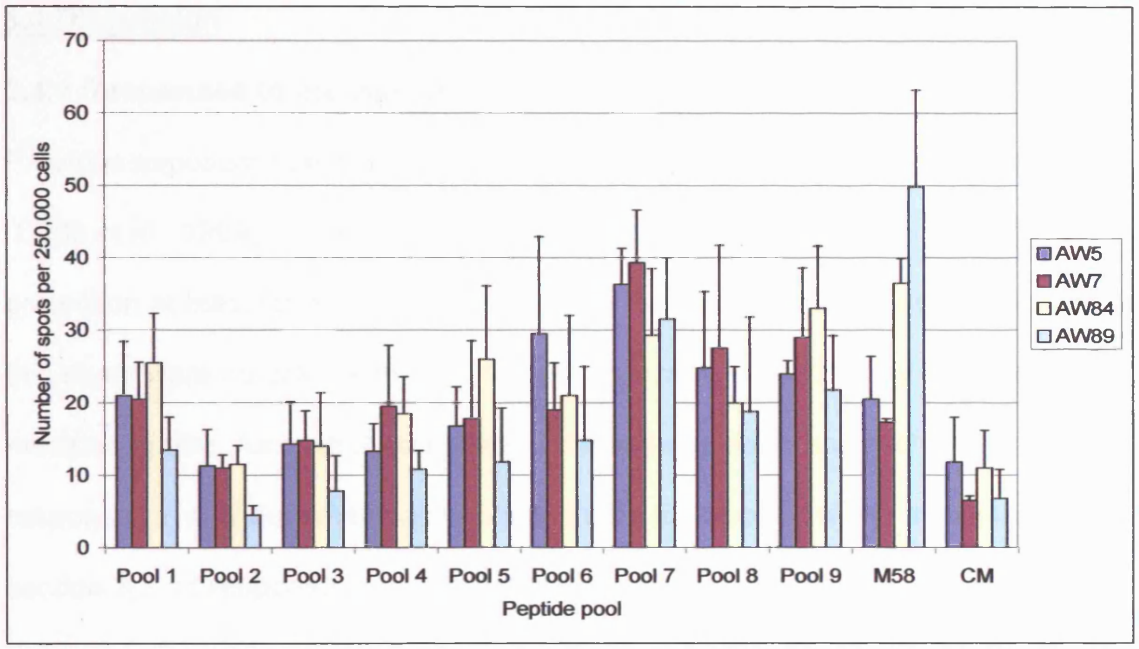


Figure 3.18: Responses to pooled MV F protein peptides of 4 MV immune HLA-A2 positive healthy adult donors in a standard ELISpot (mean of 3 wells +/-1SD). Compared with the M58 positive control peptide all peptides were used at a concentration of 10μM.

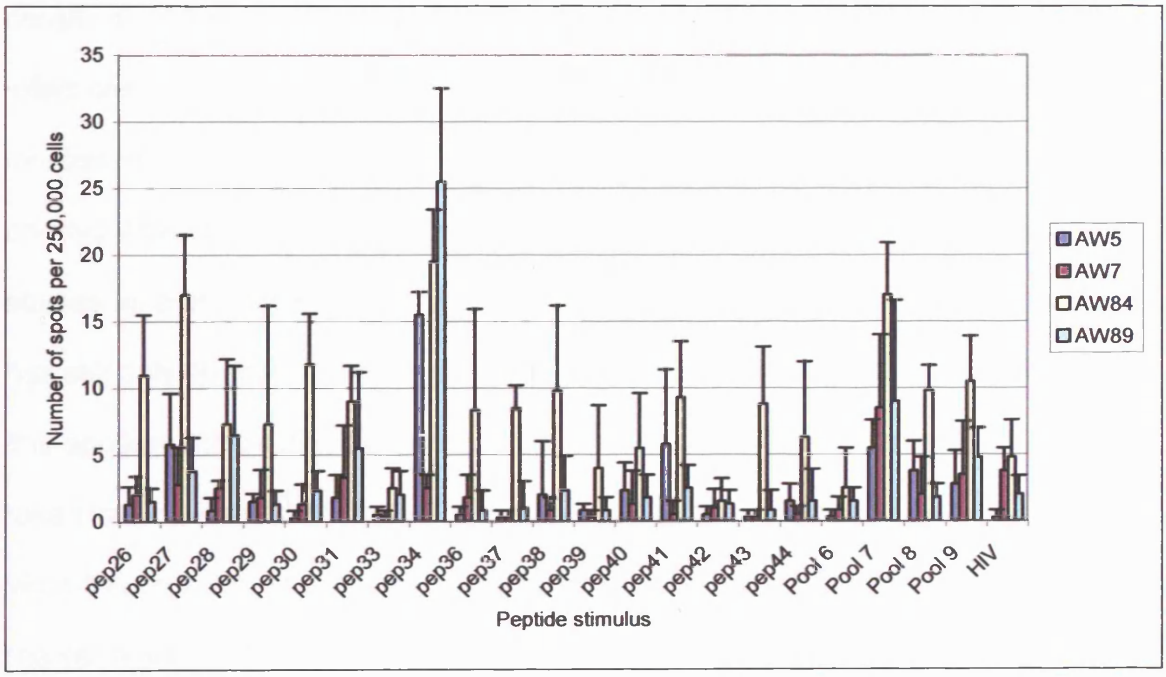


Figure 3.19 Responses to split pools of MV F protein peptides in 4 MV immune HLA-A2 positive healthy adult donors in a standard ELISpot (mean of 3 wells +/-1SD). Compared with the HIV negative control peptide all peptides were used at a concentration of 10μM.

3.4 Discussion

3.4.1 Responses to measles peptides in the ELISpot assay

Previous exposure to measles is thought to induce lasting immunity to measles (Cutts et al., 1999). Re-exposure does not appear to be necessary to maintain protection at least for wild type measles (see section 1.3.1). The donors chosen for this study were naturally immune HLA-A2 donors as measured by a positive anti measles Ig titre. As such it was predicted that these donors would give a positive response to individual measles peptides in the ELISpot, however as shown in section 3.3 no response could be detected.

The lack of detection of antigen specific T cells could be due to several factors. The precursor frequency of MV antigen specific T cells could be too low for the sensitivity of the assay, possibly due to the current situation in the UK where MV infections are rare and so there is minimal natural boosting of T cell numbers. As discussed in section 1.2.7 typical precursor frequency of CTL specific for non chronic viral infection is quite low. For example the influenza peptide used in these studies as a positive control, is the most abundantly recognised epitope however has still only 40 responders per million PBMC (Rehermann et al., 1996) although this appeared to be slightly higher in the individuals tested in this study. When taken into consideration that individuals will be regularly boosted with circulating virus it seems that the precursor frequency of CTL to MV epitopes may be below the sensitivity of the ELISpot assay. Furthermore, due to the central/peripheral memory dichotomy discussed in chapter 1, it is possible that a large proportion of memory cells may not be detected after only 16 hours in an ELISpot (which measures rapid production of IFNγ) as the central memory cells will take longer to

mobilise. Use of other techniques such as the longer proliferation assay, may be a solution to this. This method was not investigated in the first instance however due to the lack of exact quantitation of precursor responder cell frequency that this method provides.

3.4.2 Improving in vitro responses to MV peptides

As shown in section 3.3, the efficiency of antigen presentation in the ELISpot assays was raised as a possible reason for the lack detectable response and was further investigated. Despite attempts to use different APC, the background responses increased as much as the positive controls and measles peptides, making these strategies unhelpful in improving detection of responses. Using autologous DCs however did seem worth pursuing as a line of investigation (see section 3.3.4.1). DCs have been used to amplify detection of other T cell responses in vitro, such as responses to adenovirus (Leen et al., 2004) although this was done by infecting DCs with adenovirus which due to the cytopathic effects of MV on human DCs as discussed in section 1.3.8 is not a viable strategy for expanding T MV specific T cells. DCs were not used in the first instance due to the initial project aim of looking at and quantifying responses in small paediatric blood samples, which would not allow for the production of DCs. However this strategy could be utilized in future studies aimed at identifying responses to specific peptides in adults, prior to quantification of these responses in vaccinated children.

3.4.3 Responses to MV peptide pools

Following the finding that no response could be detected to the predicted epitopes despite some being identified in other assays to detect T cell memory, there was

the possibility that the cells that responded to these peptides, responded by proliferating rather than the instant effector function of producing IFNγ. Thus overlapping peptides spanning 3 of the major MV proteins were used in a more general approach to scanning for potential epitopes. Although some pools gave positive results, when the pools were split, most of them failed to identify a dominant peptide within the pool. Where a peptide was identified there was no obvious sequence that would bind in the groove of the HLA-A2*0201. Thus the way this work could be extended would be to synthesise overlapping 9mers within the peptides identified. The disappointing lack of obvious responses seen to the peptide pools again raised the issue that precursor frequencies within the individuals tested were too low for the sensitivity of the assay. Thus the next line of investigation involved the decision to vaccinate two of the HLA-A2 positive, MV seropositive individuals with the MMR vaccine, in an effort to boost the responses seen to the MV peptides *in vivo*. This strategy would also allow the monitoring of any changes in responses over time to assess if the predominant responses were the same straight after vaccination compared to 6 or 12 months later. This work is detailed in chapter 4.

Chapter 4 In vivo boosting of responses to MV through vaccination

4.1 Introduction

A problem that may have been encountered when scanning for MV T cell epitopes (as described in chapter 3), was that the approach taken sought to detect responses in healthy immune individuals who may not have encountered antigen recently and therefore may not have been boosted for many years. Thus the levels of precursor T cells may have been below the detection levels of the assay. It is well known that for other viruses CTL precursor frequency may be very low even when immunity is effective and the individual may encounter antigen more regularly than with MV. For example, in Hepatitis C virus (HCV) responses to one epitope are thought to be in the region of 10 responders per million PBMC compared to up to 40 responders per million PBMC against the Influenza matrix peptide (Rehermann et al., 1996) (this is discussed in section 1.2.7). Given that the sensitivity of the ELISpot has been shown to be close to 100% when detecting beads and specific numbers of T cell clones (Schmittel et al., 1997), the detection limit of the assay is potentially 1/250,000 cells (the number of cells per well) depending on the background (non specific spot formation). In this system on average 3 non specific spots are seen in each well therefore statistically for a stimulation index of 3, 9 spots would have to be detected to be considered significant which requires 6 spots per 250,000 cells -24 per million to be antigen specific and this is about half the level of responders seen for influenza, a virus individuals encounter much more often than MV.

Therefore in order to boost responses to MV, two volunteers were vaccinated with the MMR and sequential blood samples were taken and PBMC stored for analysis for response to the MV peptide pools. Wong-Chew et al have studied the effect of boosting previously immune adults with a measles vaccine (Wong-Chew et al., 2003). This study showed a transient boost in T cell proliferation levels to MV antigen compared to no proliferation prior to vaccination (this despite high MV specific Ig titres prior to vaccination). This was therefore encouraging for the line of investigation used here. CFSE labelling of PBMC drawn at different time points post vaccination was used to measure proliferation of T cells to MV antigen.

4.2 Specific materials and methods

4.2.1 Vaccine and volunteers

The volunteers were fully consented and given one dose of the Aventis Pasteur MMR vaccine M.M.R. II in the presence of a physician and in a clinical area. The vaccine contains 1,000 tissue culture infectious doses 50 (TCID) of live MV, Enders line of the Edmonston strain, 20,000 TCID50 of live mumps, Jeryl Lynn Level B strain and 1,000 TCID50 of Rubella, Wistar RA 27/3 strain. The vaccine was obtained through Great Ormond St Hospital pharmacy. Volunteers were allocated linked anonymised codes, MMR01 and MMR02 for labelling of all laboratory samples. Both volunteers were HLA-A2*0201 positive adult males (as confirmed by PCR genotyping) they also shared the alleles B*07 and C*0702. They were 45 and 32 years old respectively. MMR01 had had MV as a child approx 40 years prior to this study. It is assumed that MMR02 has also suffered from MV as a child however could not remember. Neither of the volunteers had been previously immunized against MV.

4.2.2 Bleeding schedule

50ml of blood was taken on each of the days indicated below in Figure 4.1, the PBMC were extracted and stored in $7.5x10^6$ aliquots in liquid nitrogen.

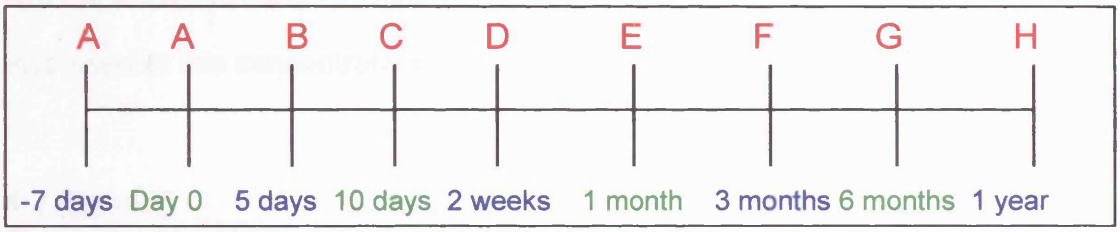


Figure 4.1 Bleeding schedule for MMR study (not to scale). Letters represent the codes that were allocated to the different time points e.g. pre vaccination MMR01A, 2 weeks post vaccination, MMR01D.

4.2.3 Production of MV antigen

MV antigen was produced by infecting a 75% confluent Vero cell monolayer with MV Edmonston –Zagreb strain which is the attenuated MV strain used for vaccination (Garly et al., 2001) obtained from the National Collection of Pathogenic Viruses (NCPV) at a multiplicity of infection (number of virus particles per cell) (MOI) of 0.1. This was done by adding 10ml of virus containing serum free medium to the Vero cells and incubating for 2 hours at 37°C/5%CO_2. This inoculum was then replaced with 100ml of culture medium containing fetal calf serum (this was in a T225 flask). The viral antigen was harvested when the cytopathic effect of the virus as observed by standard microscopy was at 80-90% indicated by syncytium formation. This typically took 2-3 days at 37°C/5%CO_2 . Cells were harvested by scraping them off the flask, which resulted in approx 2.5×10^5 cells per ml of medium which was then ruptured by 6 rounds of freeze thawing, before the suspension was spun at 1000g for 5 minutes, aliquoted and frozen at -80°C until use. In parallel with this preparation, uninfected cells were prepared in the

same way as a negative control for the presence of Vero cell derived proteins in the virus preparation. Proliferation to MV antigen was measured using CFSE labelling of cells as described in section 2.5.5. The MV antigen preparation was used at a concentration of 1/25 of the culture medium, the vero control antigen was also used at this concentration.

4.3 Results

4.3.1 Serology of individuals at different time points

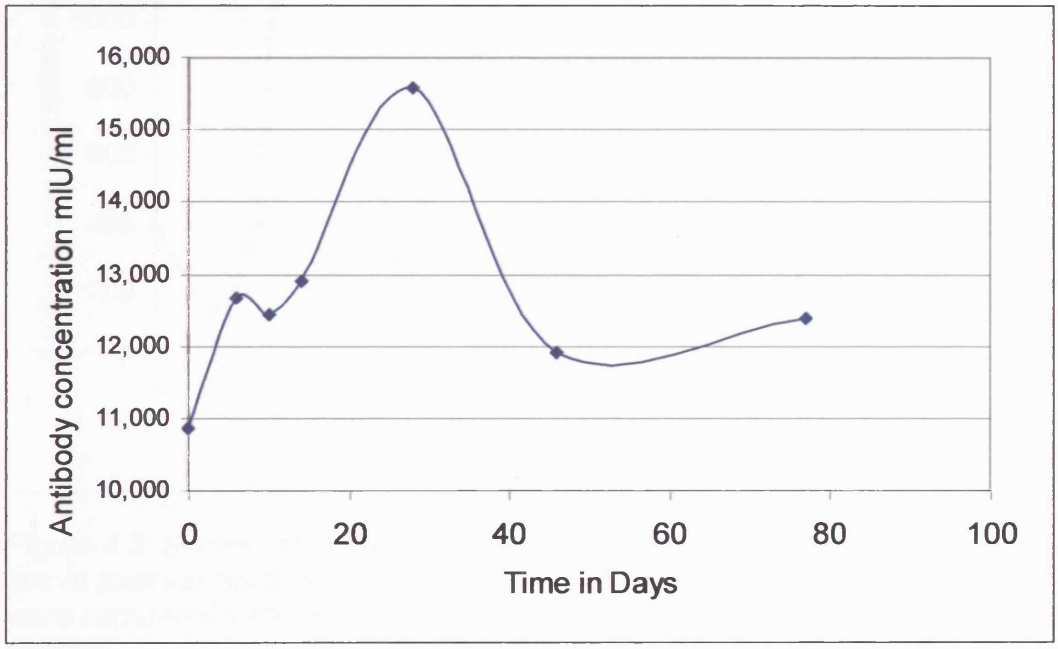


Figure 4.2: Serum anti measles IgG antibody titres in MMR01 pre and at time points post vaccination. Titres were measured in an ELISA against MV antigen and were compared with the WHO standard to give values in International Units (IU/ml).

In order to determine the MV immune status of volunteers, anti MV IgG antibody was measured for all the time points from both of the individuals vaccinated as shown in figures 4.2 and 4.3 below. This was done as outlined in section 3.2.2 by

comparing ab concentrations with an international standard in an ELISA. Both individuals were considered immune before vaccination and both show a transient boost in antibody levels around 30 days after vaccination. However the antibody levels are ten fold different between the two volunteers showing significant variation between individuals.

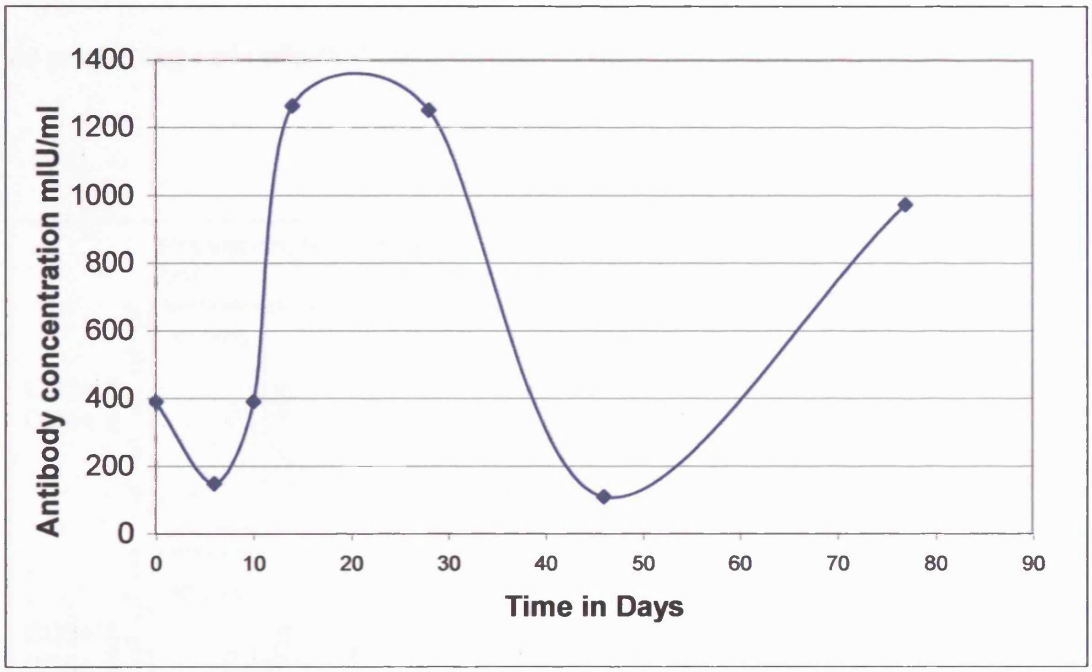


Figure 4.3: Serum anti measles IgG antibody titres in MMR02 pre and at time points post vaccination. Titres were measured in an ELISA against MV antigen and were compared with the WHO standard to give values in International Units (IU/ml).

4.3.2 Proliferation to whole measles

Whole measles antigen was prepared as outlined in section 4.2.3 and was compared in all proliferation experiments to Vero cell lysate prepared identically except without MV infection. Responder cells were labelled with CFSE and incubated with Vero cell lysate for 5 days, before being harvested and stained for CD3, CD8 and CD4. Plots shown in figure 4.4 are gated on live, CD3 positive cells

and the percentages shown are the percentage of CD3+, CD4+ or CD8+ cells that have proliferated to the antigen. Only the donor MMR02 is shown for the time points pre and 6 months post vaccination in the FACS plots however the same pattern is repeated in MMR01 where there is less proliferation to MV antigen post vaccination than before. Proliferation to MV at all time points post vaccination for both MMR01 and MMR02 is summarised in figure 4.5, in which data are expressed as percentage of cells that have divided in each sub population.

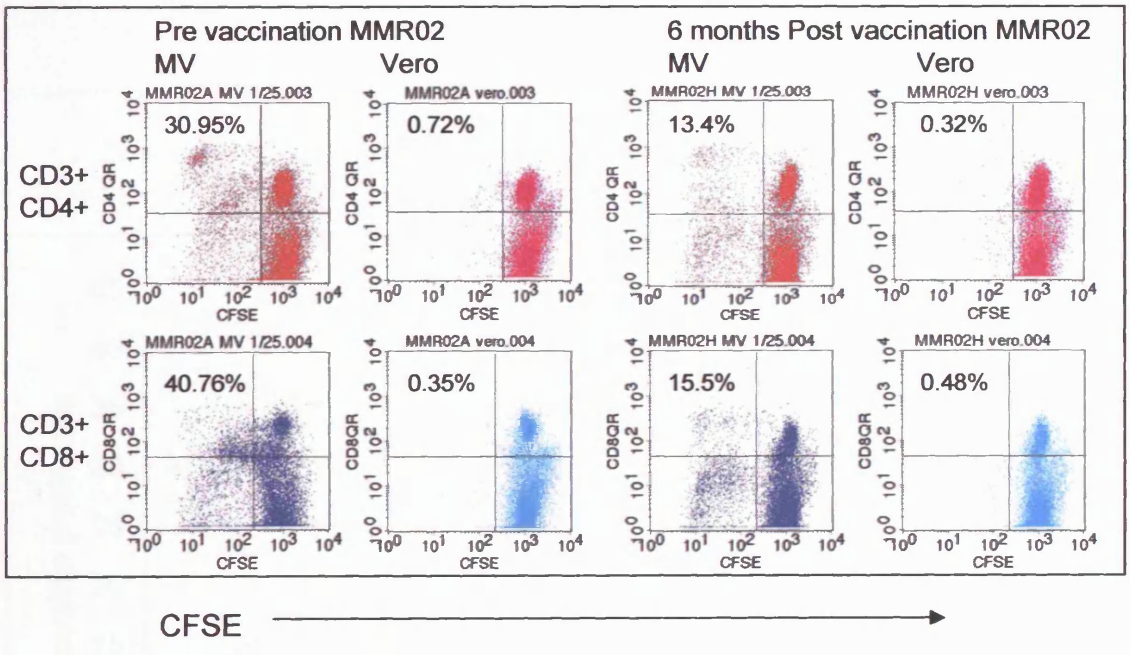
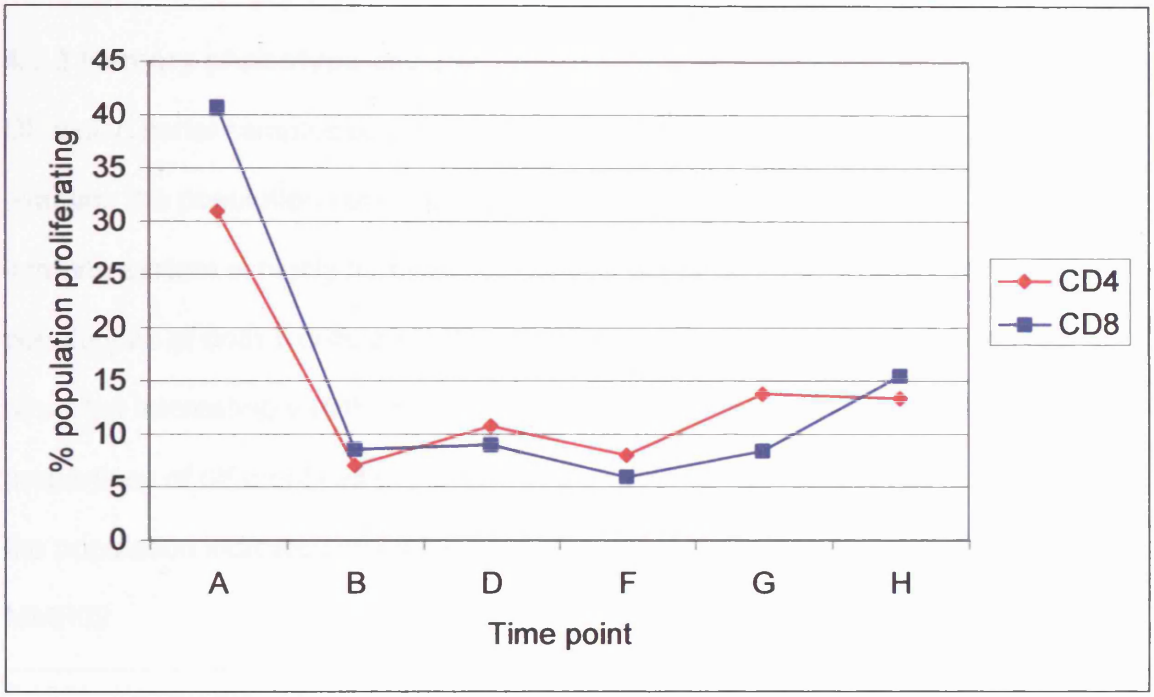


Figure 4.4: Proliferation of T cell subsets to MV antigen (CD3+CD4+ upper row, red proliferation to MV pink to control, CD3+CD8+ lower row blue proliferation to MV turquoise to control) to MV antigen or uninfected Vero control from volunteer MMR02 pre (left four panels) and 6 months post (right four panels)vaccination.

a)



b)

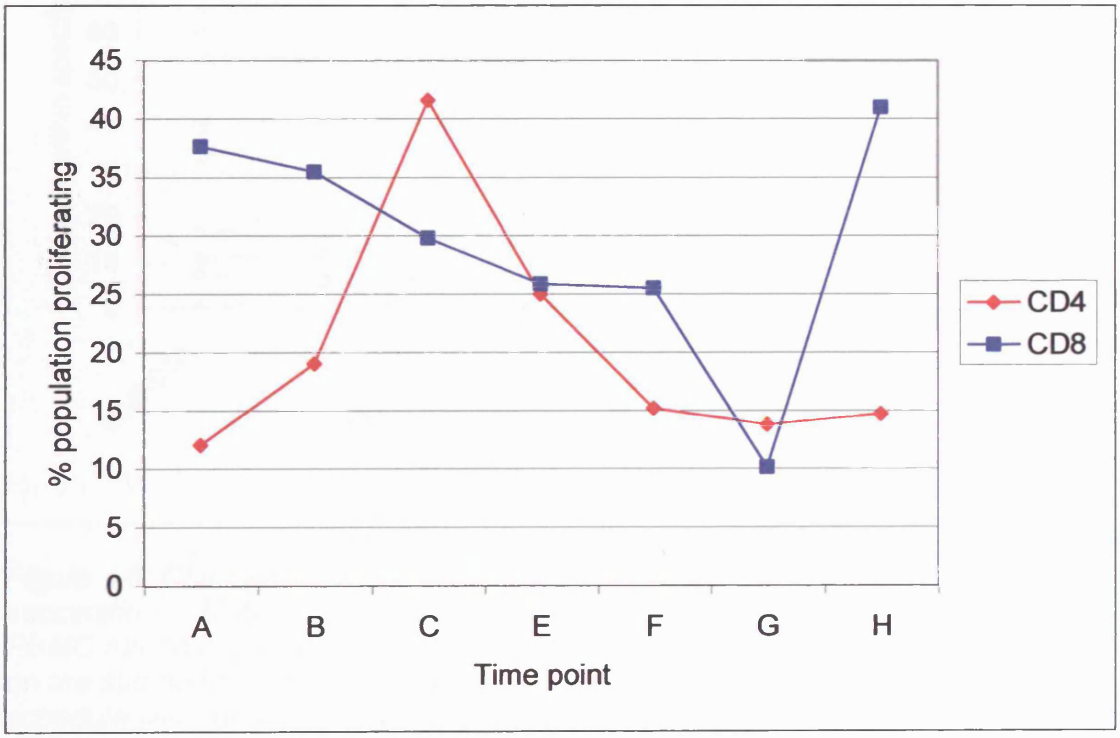


Figure 4.5 Graphical representation of proportions of CD3+CD4+(red) and CD3+CD8+(blue) T cells proliferating to MV antigen over time post vaccination in a)MMR01 and b)MMR02.

4.3.3 Memory phenotype analysis

Obtaining serial samples post vaccination provided a unique opportunity to examine the populations of cells in the PBMC after vaccination. Although the immune system is highly homeostatic the use of flow cytometry to detect distinct populations of both T cells and other peripheral cell populations showed some small but interesting variations in frequency. Figures 4.6 and 4.7 show the proportions of different cell populations as a percentage of either total cells or of the population indicated changing over time post vaccination in both MMR01 and MMR02.

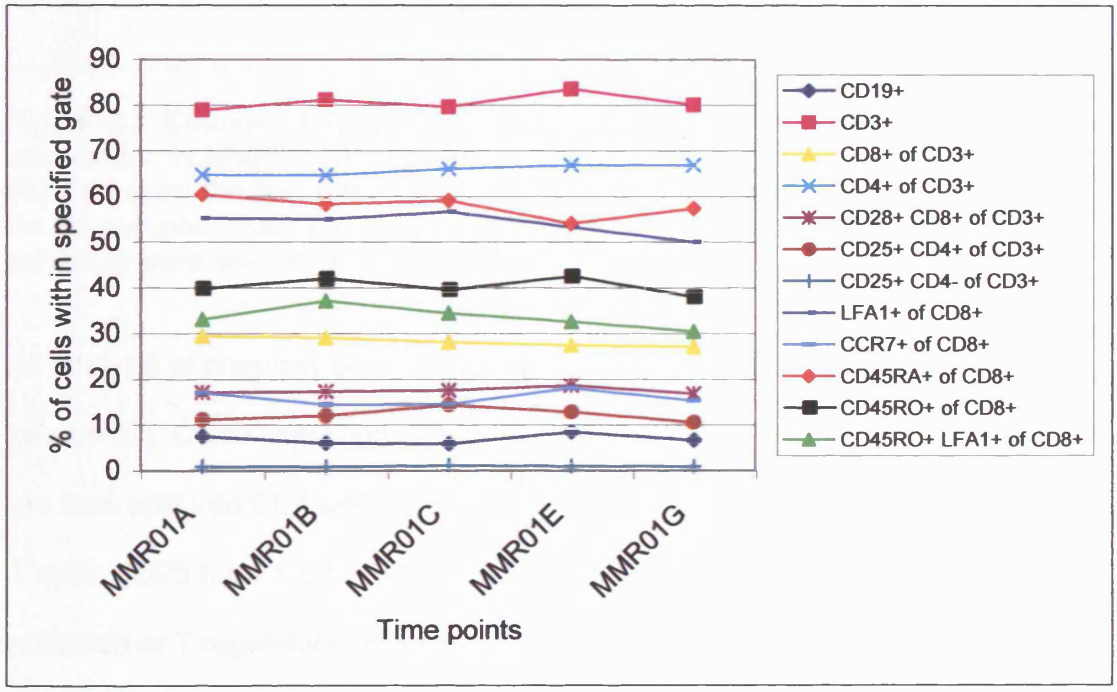


Figure 4.6: Changes in lymphocyte sub populations in PBMC over time post vaccination in MMR01. Data generated by analysis of three colour staining of PBMC run on the flow cytometer. Cells were gated on a lymphocyte gate and then on the sub population indicated in the legend. Five time points in the vaccination schedule were analysed (as shown in figure 4.1).

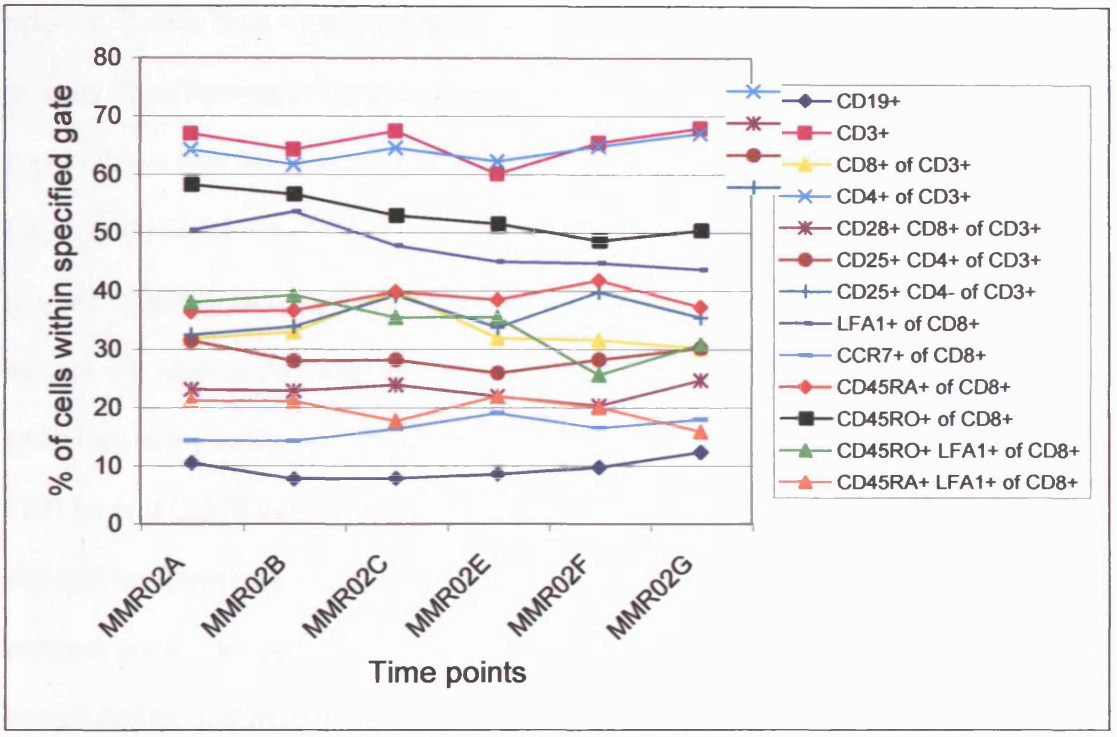


Figure 4.7: Changes in lymphocyte sub populations in PBMC over time post vaccination in MMR02. Data generated by analysis of three colour staining of PBMC run on the flow cytometer. Cells were gated on a lymphocyte gate and then on the sub population indicated in the legend. Five time points in the vaccination schedule were analysed (as shown in figure 4.1).

All analysis of phenotypes was carried out using flow cytometry as outlined in section 2.5. CD19 was used to stain B cells and CD3 to stain T cells. The T cells are then split into CD4 and CD8 positive cells indicating T helper cells or Cytotoxic T cells. CD25 high, CD4 positive cells could indicate a population of recently activated or T regulatory cells in the CD4 population (Kuniyasu et al., 2000). The CD8 population is then split into two cell populations expressing either the CD45 isoform CD45RA or CD45RO. These two populations at one time were believed to represent either naïve T cells (CD45RA) or antigen experienced cells (CD45RO). However recent studies both using TCR analysis and MHC tetramer staining have shown that the CD45RA+CD8+ population contains high numbers of antigen

specific T cells thus must contain memory cells (Dunne et al., 2002). Further studies investigating integrin and chemokine receptor surface expression on cells have shown that the antigen experienced CD45RA+ cells express high levels of LFA1 and low levels of CCR7. This indicates a population of cells that home to tissues rather than lymph nodes (CCR7 being a receptor crucial for extravasation across the high endothelial venule (HEV) (Dunne et al., 2002;Faint et al., 2001), therefore entry to the lymph nodes. LFA1 is an adhesion molecule made up of CD11a and CD18 that allows for homing to tissues, thus although the cells exhibit the low turnover rate of naïve cells they home to tissues and can be activated on antigen encounter without the need for professional antigen presentation in the lymph nodes and may then become RO positive effector cells. As can been seen in figures 4.6 and 4.7, the populations do vary with time post vaccination in some cases up to 7 or 8% of a sub population. How much significance this has *in vivo* is hard to determine, and more individuals would need to be studied to determine a pattern and by comparison with variation over time in non-vaccinated controls. However in these two individuals it can be seen that although there is little change in the levels of CD3+CD4+ cells in one individual (MMR02) the proportion of CD3 cells that are CD8 positive increases 10 days post vaccination from 31% to 40%. In the same individual there is also an increase in CD4+CD25+ cells which may indicate the presence of a regulatory population beginning to control the response at the 10 day time point or a population of activated, dividing cells up-regulating the IL-2 receptor. Perhaps more interestingly the percentage of CD8+ cells that are LFA1+ in both individuals, decrease at the 10 day time point as does the LFA1high CD45RO population which could be activation induced cell death of the effector

cell population. It is also interesting to note the relationship between RO and RA cells being that when one increases the other decreases.

4.3.4 T cell responses to pools of MV peptides assayed by ELISpot

In order to investigate the MV peptides to which the MMR vaccinated individuals responded, and to demonstrate any similarities between the two donors over time, the pools of peptides from the three MV proteins NP, H and F (tables 3.2, 3.3 and 3.4) were used to stimulate PBMC from all time points in the ELISpot assay. Where possible pools were then separated into their individual peptides and used to stimulate PBMC from specific time points, to try to identify specific peptides that were stimulating IFNγ release. In order to allow easier comparison over time values have been normalized by subtracting the number of spots in the control wells at each time point and are expressed as spots per 250,000 cells per well. The results of IFNγ release in response to peptides derived from the MV NP protein are shown in figures 4.8 (MMR01) and 4.9 (MMR02).

4.3.4.1 Response of vaccinated individuals to peptides derived from MV NP protein

As can be observed in figures 4.8 and 4.9 there is a trend towards an increase in response to pool 1 and 2 in both individuals with pool 1 peaking late in the response at time point F (3 months) in MMR01. Responses to pool 4 appear to increase gradually in both donors peaking late at time point E (1 month). In MMR01 responses to pool 5 peak at time point C (10 days) but not in MMR02. Responses to pool 8 rise at 2 weeks in MMR01 and 1 month in MMR02. Responses to pool 9 gradually increase over the time to peak at 1 year and those to pool 10 peak at 1

month in both donors. These data, especially where both donors show the same pattern of response highlight pools and time points that warrant more investigation by splitting the pools into their individual peptides and testing responses to these (see section 4.3.5).

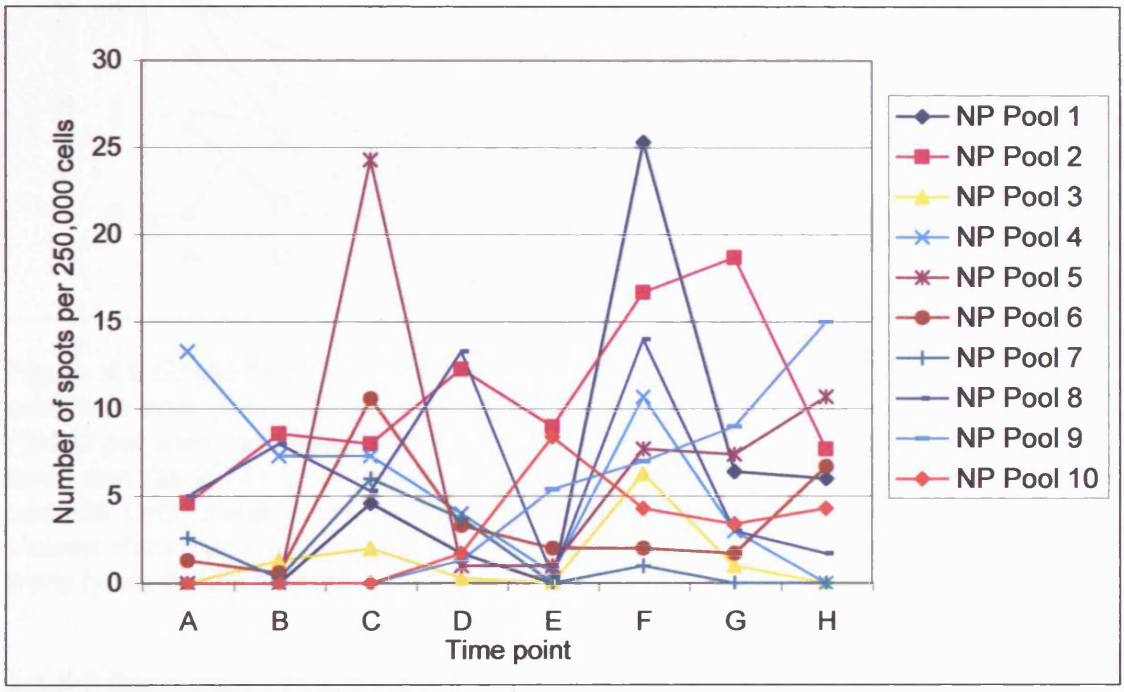


Figure 4.8 Graph to show the response of MMR01 PBMC to MV NP peptide pools over time post vaccination. Standard ELISpots were carried out with 250,000 PBMC per well stimulated with 10μM of each of 5 peptides per pool. Values have been normalized to responses at each time point to the negative control HIV peptide. Letters represent the time points post vaccination as outlined in figure 4.1. Values are mean of 3 wells SD are not shown for ease of viewing but were typically as low as in previous experiments.

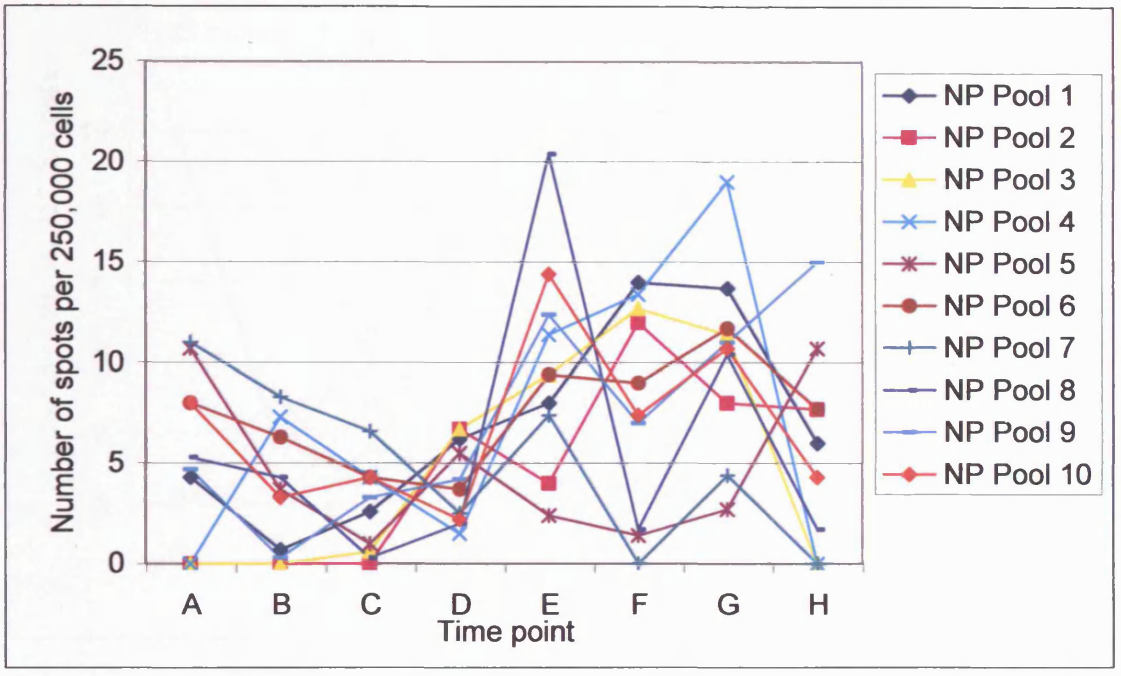


Figure 4.9 Graph to show the response of MMR02 PBMC to MV NP peptide pools over time post vaccination. Standard ELISpots were carried out with 250,000 PBMC per well stimulated with 10μM of each of 5 peptides per pool. Values have been normalized to responses at each time point to the negative control HIV peptide. Letters represent the time points post vaccination as outlined in figure 4.1. Values shown are mean of 3 wells, SD are not shown for ease of viewing however were typically low as in previous experiments.

4.3.4.2 Response of vaccinated individuals to peptides derived from MV H protein.

As can be seen from figures 4.10 and 4.11 pool 1 of the H peptide pools appears to be the most interesting as it is consistently giving high responses in MMR02 and appears to peak at time point D (2 weeks post vaccination) in MMR01. The response to pool 2 appears to peak around 2 weeks post vaccination in both donors making this a potentially interesting pool to investigate further. Responses to pool 7 peak late in both donors with a steady increase in response in MMR02. Responses to pool 9 also have an interesting pattern over time in both donors, with a peak response at 2 weeks which then gradually declines post vaccination.

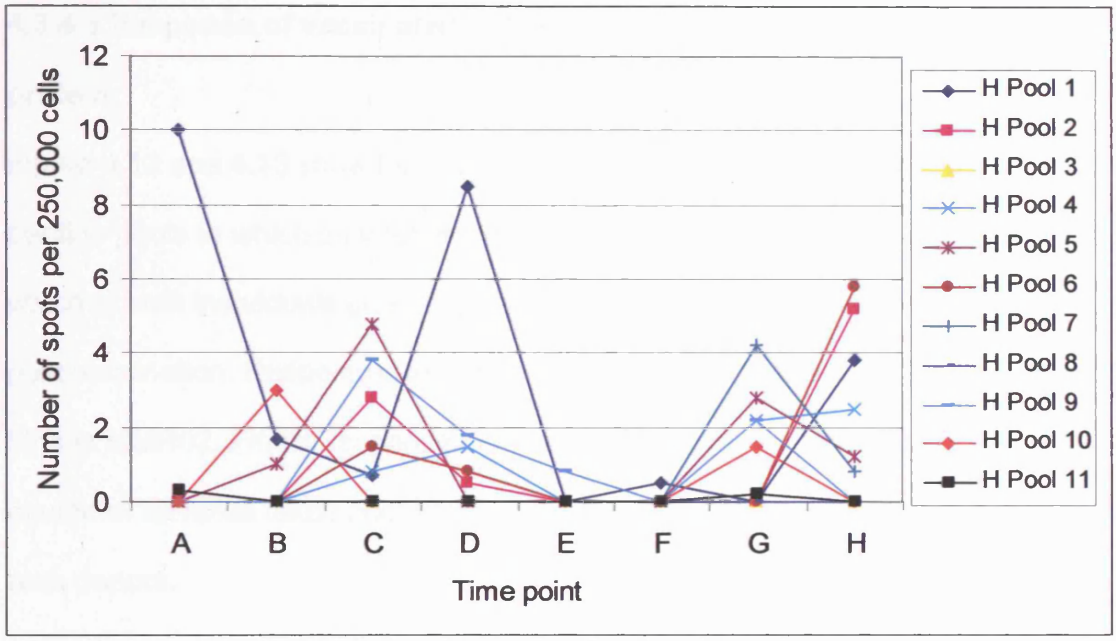


Figure 4.10 Graph to show the response of MMR01 PBMC to MV H peptide pools over time post vaccination. Standard ELISpots were carried out with 250,000 PBMC per well stimulated with 10μM of each of 5 peptides per pool. Values have been normalized to responses at each time point to the negative control HIV peptide. Letters represent the time points post vaccination as outlined in figure 4.1.

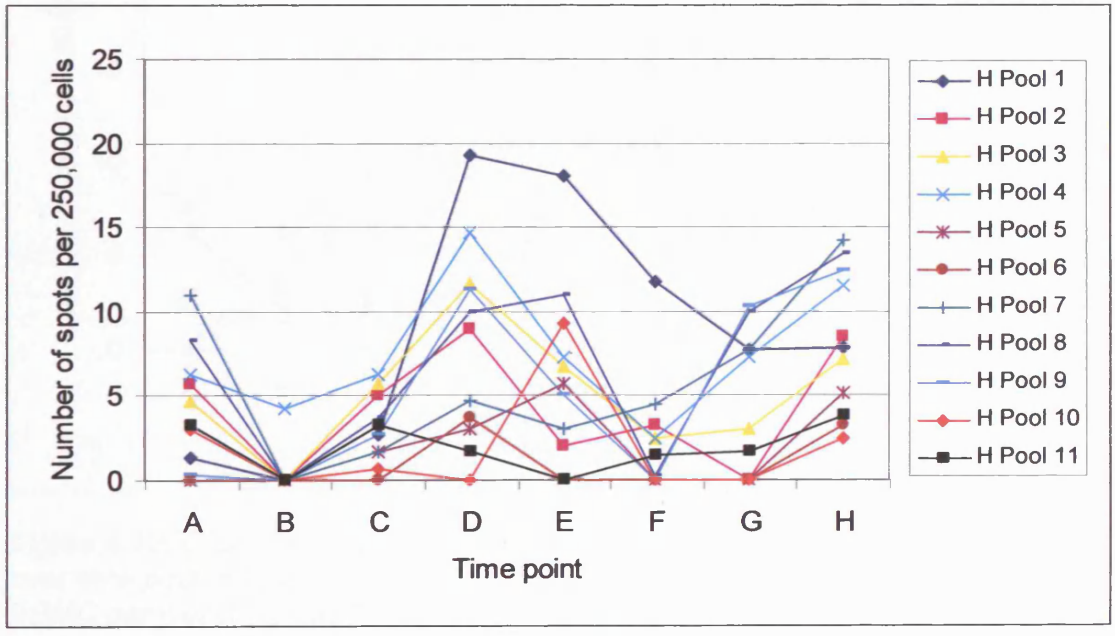


Figure 4.11 Graph to show the response of MMR02 PBMC to MV H peptide pools over time post vaccination. Standard ELISpots were carried out with 250,000 PBMC per well stimulated with 10μM of each of 5 peptides per pool. Values have been normalized to responses at each time point to the negative control HIV peptide. Letters represent the time points post vaccination as outlined in figure 4.1.

4.3.4.3 Response of vaccinated individuals to peptides derived from MV F protein.

Figure 4.12 and 4.13 show that as for the NP and H, proteins there are several peptide pools to which an interesting pattern of response was seen. Firstly pool 1 which in both individuals gives high responses, which in MMR02 peaks at 2 weeks post vaccination. Responses to pool 7 have a peak at 2 weeks and are consistently high in MMR02. Finally responses to pool 10 rise towards the end of the time course of samples taken post vaccination (time point H 1 year post vaccination) in both donors.

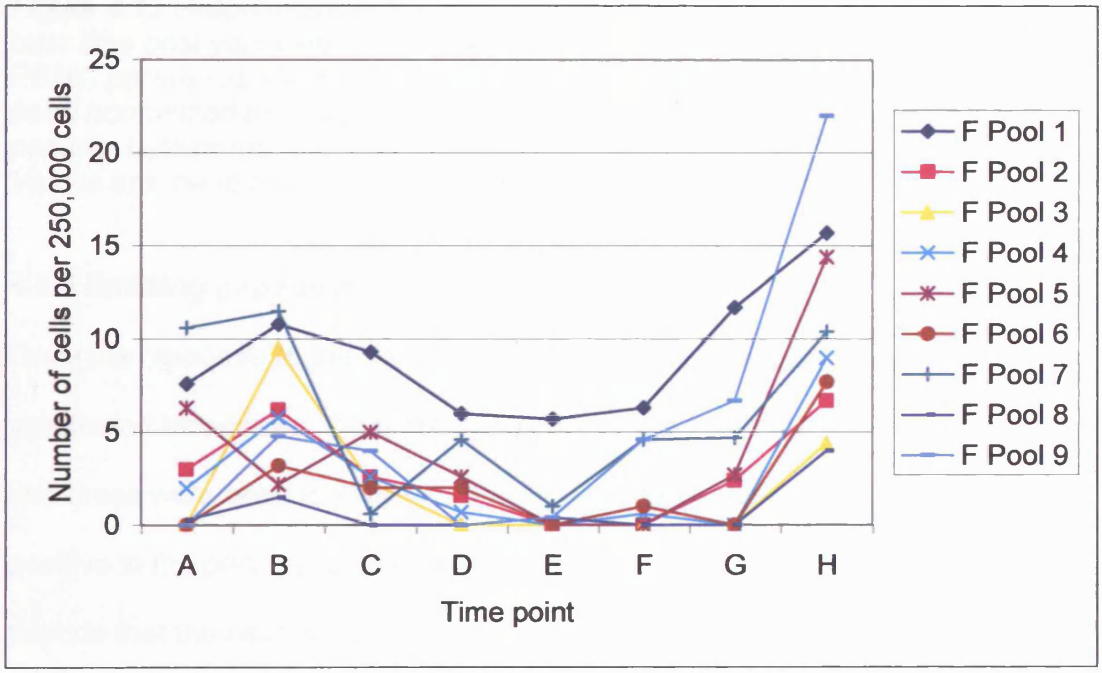


Figure 4.12: Graph to show the response of MMR01 PBMC to MV F peptide pools over time post vaccination. Standard ELISpots were carried out with 250,000 PBMC per well stimulated with 10μM of each of 5 peptides per pool. Values have been normalized to responses at each time point to the negative control HIV peptide. Letters represent the time points post vaccination as outlined in figure 4.1. Values are means of 3 wells, SD not shown for ease of viewing.

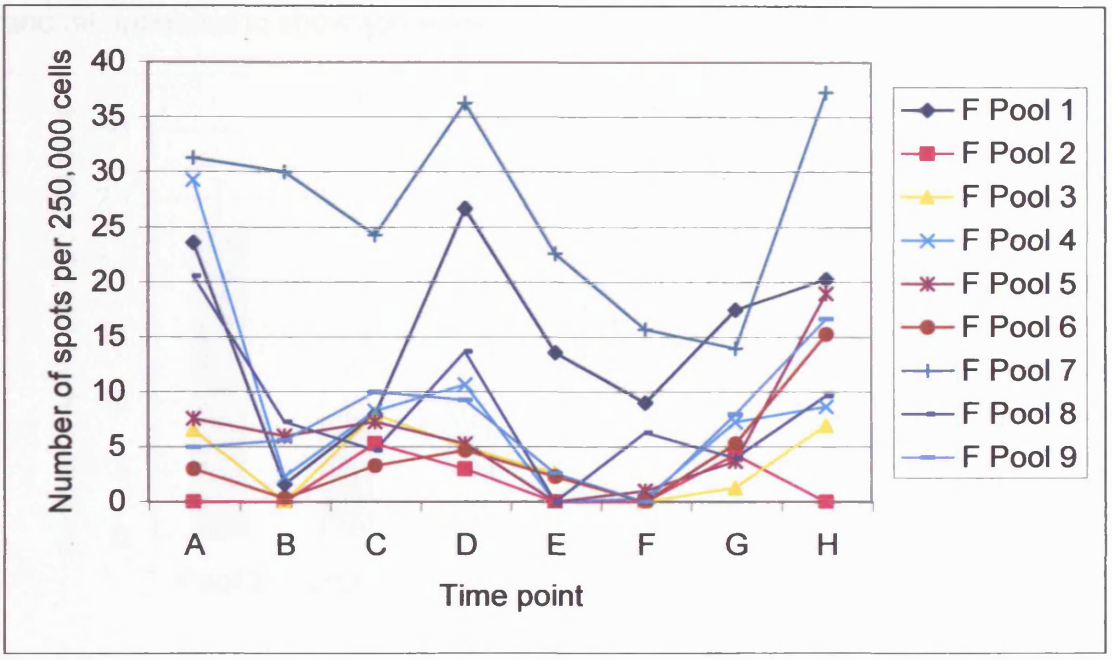


Figure 4.13 Graph to show the response of MMR02 PBMC to MV F peptide pools over time post vaccination. Standard ELISpots were carried out with 250,000 PBMC per well stimulated with 10μM of each of 5 peptides per pool. Values have been normalized to responses at each time point to the negative control HIV peptide. Letters represent the time points post vaccination as outlined in figure 4.1. Values are mean of 3 wells, SD not shown for eas of viewing.

4.3.5 Splitting peptide pools

Once the response to the MV derived peptides pools had been investigated in the vaccinated individuals, the highlighted pools were then split into individual peptides and these were used to stimulate PBMC from the time points that had been positive in the previous experiments, in an ELISpot, in order to identify a specific peptide that the cells were responding to. Due to the limited amount of cells in the samples available from the volunteers, this could only be done for the NP protein pools in the first instance. The results of this are shown in figures 4.14 and 4.15. For MMR01 the time points and NP derived peptide pools that were investigated were, C- pool 5, D pools 2 and 8, E pool 10, F pool 1 and G pool 2. Peptides 22

and 38 appeared to show some response over background (figure 4.14)

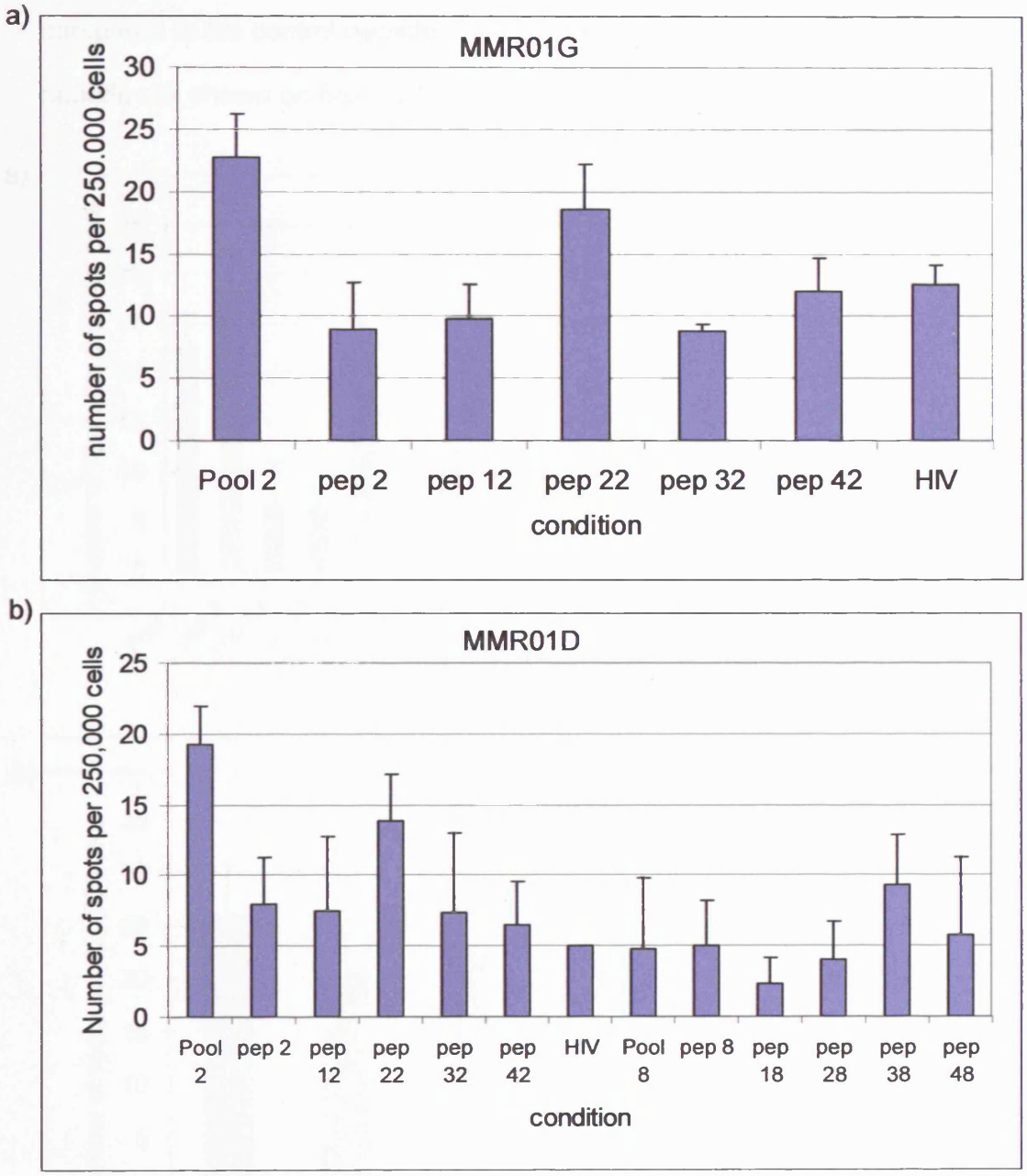


Figure 4.14: MMR01 PBMC simulated by split MV NP peptide pools, a) MMR01G with split pool 2 b) MMR01D with split pools 2 and 8. Standard ELISpots were carried out where PBMC were stimulated with 10μM of individual MV NP peptides from identified peptide pools. A positive response was considered to be more than a doubling of responses to control HIV peptide which is shown for comparison. Values represent mean of 3 wells +/- 1SD.

Response to the peptides was defined by at least a doubling of responses when compared to the control peptide. For this reason the responses to the control peptide are shown on figures 4.14 and 4.15 for comparison.

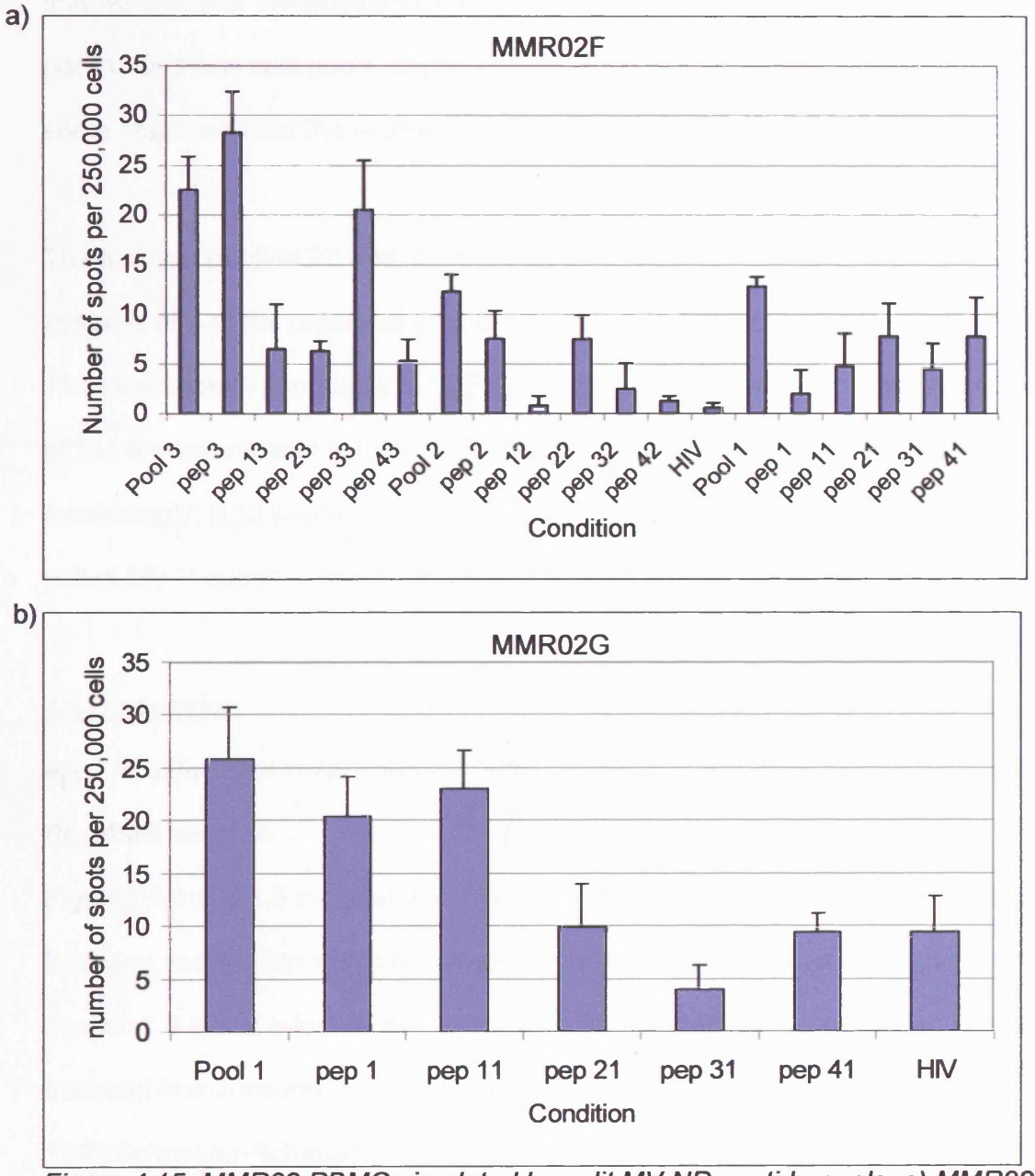


Figure 4.15: MMR02 PBMC simulated by split MV NP peptide pools, a) MMR02F with split pool 2 b) MMR02G with split pools 2 and 8. Standard ELISpots were carried out where PBMC were stimulated with 10μM of individual MV NP peptides from identified peptide pools. A positive response was considered to be more than a doubling of responses to control HIV peptide which is shown for comparison. Values represent mean of 3 wells +/- 1SD.

As these experiments could only be carried out once the increases observed were not statistically significant but served to identify individual MV NP peptides that warranted further investigation. For MMR02 the time points and NP peptide pools that were further investigated were, E -pools 8 and 10, F- pools 1 2 and 3 and G-pool 1. Of these split pools, peptides 1, 11, 3, 33, 22, 21 and 38 appeared to show some response over the response to the control peptide (figure 4.15).

The fact that peptide 22 was identified as a possible epitope was interesting as this contains one of the predicted epitopes N210 that was investigated in chapter 3. The sequence of peptide 22 is LERKWLDVVRNIIAF and the underlined section is all but one amino acid of the predicted A2 epitope N210 (RLERKWLDV). Also interestingly, H30 would be in Pool 1 in the pools of MV H peptides which is the poll of MV H peptides that is responded to most by both vaccinated individuals.

4.4 Discussion

4.4.1 Proliferative responses of PBMC to MV antigen after vaccination with the MMR vaccine

Figures 4.4 and 4.5 suggest that T cell proliferation to MV antigen is reduced following vaccination which is somewhat counter-intuitive. However as discussed in section 1.3.8, MV infection and to some extent the vaccine is associated with a transient immunosuppression which could explain these results (Hirsch et al., 1981;Schneider-Schaulies and Meulen, 1998). One study in children receiving measles vaccines showed that proliferation of lymphocytes to mitogen after vaccination was reduced both 2 weeks and three months after vaccination compared to pre vaccination levels. Interestingly it was also found that this

suppression of lymphocyte proliferation negatively correlated with the levels of anti MV antibody, therefore the stronger the response to the vaccine the more suppression of lymphocyte proliferation to mitogen (Hussey et al., 1996). The Hussey et al study did not follow the vaccinated individuals after 3 months so it is possible that this vaccine induced immunosuppression extended beyond this time point (Hussey et al., 1996). This may support the observation seen in this study where reduced levels of proliferation to MV antigen are seen up to one year after vaccination. Ideally to determine if this phenomenon is reproducible these experiments should be repeated, both to ensure that the assay reproducibility is acceptable with these samples and that the changes seen in proliferation to MV antigen are significant. In addition to this the viability of cells prior to the proliferation assay could be tested further than analysis by trypan blue, with Annexin V staining, as it may be that cells from some time points are more prone to apoptosis than others. In contrast to these data, the investigation by Wong Chew et al concluded that T cell proliferation increased post vaccination (Wong-Chew et al., 2003). However on further analysis of the methods used in the Wong study it was found that PBMC were stimulated with MV antigen but proliferation was measured by thymidine incorporation and so there was no verification that it was T cell proliferation that was being measured.

When data from this study were not gated on CD3, proliferation post vaccination was observed. However anti CD3, 4 and 8 staining showed this was not T cells proliferating. Attempts were made to identify the proliferating cells. However staining for both monocyte and B cell markers failed to identify a specific population on the small number of cells available (data not shown).

4.4.2 Memory phenotype post vaccination

In staining the PBMC stored from each time point in both vaccinated individuals it was interesting to see that some changes were detectable in the proportions of cells expressing certain markers despite the homeostatic nature of the immune system as a whole. The most interesting findings were the increase in CD4+CD25+ cells 10 days post vaccination which could indicate the emergence of a regulatory population of cells to control the response. Alternatively this could also indicate the activation and proliferation of the antigen specific cells. The decrease in CD8+ CD45RA+ LFA1+ cells post vaccination could either indicate the differentiation of these cells into a more immediate effector cell or activation induced cell death of this population due to antigenic stimulation. Due to the small numbers of vaccinees, it is impossible to determine how much significance these changes have, however this pilot study shows that it may be an interesting area to study in the context of larger vaccine trials in order to further characterize the effect of vaccination and further investigate the immune response as a whole. This could be then compared to the studies discussed in section 1.3.7 which describe the immune responses to wild type MV infection.

4.4.3 Dissecting peptide pools

Although several epitope candidates arose from this analysis, only two of the MV NP peptides produce a response in both MMR01 and MMR02. By using 15mer overlapping peptides the study might have identified not only HLA-A2 binding epitopes but also of identifying epitopes that bound to other HLA molecules on the surface of the cells including Class II epitopes. However as this study was

restricted to just 2 individuals, it was decided to focus in on the original aim of identifying HLA-A2*0201 epitopes.

The two peptides that were responded to by both MMR01 and MMR02 were NP peptide 22 and peptide 38. Peptide 22 contains the initial N210 peptide that was investigated in chapter 3. On further investigation it was found that the initial predicted epitope H30 would be in Pool 1 in the pools of MV H peptides which is the H pool that is responded to most by both vaccinated individuals. This would be interesting to further investigate in the context of recently vaccinated individuals in future studies.

The other NP peptide identified by splitting the pools into individual peptides was NP 38 which has the sequence RSAGKVSSTLASELG this does not contain any immediately apparent A2 motif sequences. However both MMR01 and MMR02 share two other MHC alleles, B*07 and C*0702, the binding motifs for which are XPXXXXXXL/F and XXXXXXXXY/F/L respectively. There are no potential binding sequences for B*07 in either peptide 38 or peptide 22 however both peptides contain sequences that may bind to the C*0702 allele, SSTLASEL for peptide 38 and DVVRNIIAF for peptide 22.

With regards to the initial predicted epitopes that had been identified by various techniques as outlined in chapter 1, it appears that the vaccinated individuals do respond to two of these peptides by producing IFNγ. H30 was initially identified by Nanan and colleagues (Nanan et al., 1995;Nanan et al., 2000) firstly by proliferation of T cells to the peptide and then by production of IFNγ. It was also

identified as a naturally processed epitope by MV infected B cells by van Els and colleagues (van Els et al., 2000). N210 was also identified by proliferation to the peptide by Nanan et al. It is important that these epitopes have been verified by other methods and it will be interesting to investigate the response to these epitopes in newly vaccinated naïve individuals and in natural measles infection in order to further characterize the immune response to measles.

Although this method did identify several potentially interesting peptide sequences the responses seen were still very small and a slightly higher background spot level could change the interpretation of the data. This suggests that even after vaccination the precursor frequency of the CTL specific for these epitopes is very low. Even the largest responses over background were only 10 spots in 250,000 cells which is 40 per million PBMC. This is within the same precursor frequency as the influenza matrix protein epitope M58 as discussed in section 1.2.7. Therefore a new strategy was adopted that mirrored the strategy used by van Els et al (van Els et al., 2000) in that by infecting antigen presenting cells the result would be a naturally processed epitope. In contrast to this work however, the aim was to grow T cells lines to the APC and therefore needed to overcome the cytopathic effects that MV has on DCs. Therefore an adenoviral vector system was used to express MV proteins in DCs, this strategy is discussed in chapter 5 where autologous DCs from one vaccinated individual, MMR02 were infected with adenoviral vectors containing single MV proteins and used to stimulate the T cells in order to grow T cell lines, with the hope of amplifying the specific T cells and providing a much larger specific cell population allowing a more detailed analysis for each protein.

Chapter 5 *In vitro* boosting of responses using adenoviral constructs

5.1 Introduction

It has been shown that MV specific CTL can be detected in individuals vaccinated against MV. These were detected in a chromium release assay by killing of HLA matched allogeneic B cells (Jaye et al., 1998a). This is an assay known to correlate well with results observed in the ELISpot (Scheibenbogen et al., 2000). However in order to detect these cells it was necessary to expand them *in vitro* prior to use in these assays (Jaye et al., 1998b). Therefore it was decided to expand MV specific T cells *in vitro* prior to use in the ELISpot. It is well established that measles virus infection has a direct cytopathic effect on human dendritic cells *in vitro* and have a negative effect on T cells co cultured with these DCs this was discussed in sections 1.3.5 and 1.3.8. Therefore as an alternative strategy to provide measles antigens for presentation in *in vitro* assays, a replication deficient adenoviral vector containing cDNA coding for the full length MV NP protein was used (Rad68) alongside constructs containing full length cDNA's coding for the F (Rad95) and H (Rad88) MV proteins, or a control construct, (Rad35) containing β-galactosidase. These constructs were shown to infect human cells using low enough levels of virus to be non-toxic to the cells. It was thought if these constructs could be used to infect human DCs, this would represent a more natural route of antigen entry, since antigens are typically presented on MHC I via the intra cellular antigen presentation pathway as outlined in section 1.1.2.3. Therefore processing and presentation would more closely mirror that of natural measles, while avoiding the cytopathic effects natural measles infection would have on the cells.

Interestingly the NP protein itself has been reported to inhibit IL-12 production by DCs *in vitro* (Section 1.3.5) and may therefore have an immunosuppressive effect, which would be potentially overcome by the intracellular expression of the protein rather than it being added exogenously. These constructs would also allow the delivery of MV antigen into APC in a more natural route and therefore allow the expansion of MV specific T cells to use in further *in vitro* assays.

5.1.1 Adenovirus virology

The adenovirus is a member of the Mastadenovirus genus which infects mammals. It has a double stranded DNA genome which is encased within non-enveloped icosahedral particles which are between 60 and 90nm in diameter depending on the strain (Roy-Chowdhury and Horwitz, 2002). These are shown both schematically and in an electron-micrograph in figure 5.1.

a) b)

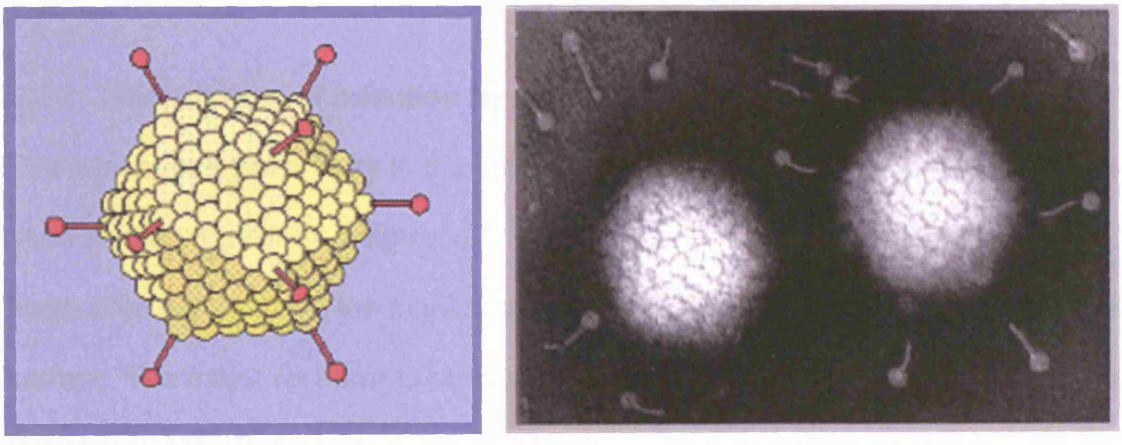


Figure 5.1: a) Schematic representation and b) electron micrograph of adenovirus particles showing the penton structures on each corner. Adapted from http://www.uct.ac.za/depts/mmi/stannard/adeno.html.

The wild type adenovirus causes mild respiratory illness and conjunctivitis in humans amongst other more serious symptoms such as gastroenteritis and pneumonia. The adenovirus family has been classified into six distinct sub groups A-F and then split further into at least 49 serotypes. This has been done on the basis of genetic variability, oncogenic potential and G-C content of DNA.(Li et al., 1999). It is subgroup C isolates that are associated with upper airway infections of which the main two are serogroup Ad2 and Ad5. Ad5 is the serogroup on which the adenoviral vectors used in this study are based, although Ad2 has also been used. The Adenovirus is ideal for use as a vector, as its life cycle does not involve integration into the genome, instead replication is as episomal elements in the host cell nucleus which means there is minimal risk of insertional mutagenesis. Adenovirus has a large genome of 35Kb, up to 30Kb of which can be replaced with foreign DNA (Verma and Somia, 1997) again making the virus an ideal candidate for a vector system.

5.1.2 Mechanisms of infection by adenovirus

The adenovirus gains entry to the cells by using the penton structure which can be seen as the projections in figure 5.1. Uptake of the adenovirus particle is a two stage process involving the binding of the penton structure to receptors on the cell surface. The major receptor to be described is the coxsackievirus and adenovirus receptor (CAR) (Bergelson et al., 1997). However at high enough viral titres adenovirus can enter cells that do not express CAR such as DCs and it is thought that MHC I may also act as a receptor (Hong et al., 1997).The penton base protein at the end of the penton spike nearest the virus particle then binds to integrins on the cell surface allowing internalisation via receptor mediated endocytosis

(Wickham et al., 1993;Wickham, 2003). Once in the phagocytic vacuole, the penton proteins have a toxic activity which is responsible for the rupture of the phagocytic membrane and release of the viral particle into the cytoplasm of the cell (Bergelson, 1999). The core of the virus then migrates to the nucleus where the DNA enters through nuclear pores where it is replicated and in wild type virus new viral particles are synthesised (Guy et al., 1995).

5.1.3 Use of adenoviral vectors for gene delivery.

With the recent increase in the use of viral vectors to deliver genes for gene therapy and anti tumour therapy (Kanerva and Hemminki, 2004;Roy-Chowdhury and Horwitz, 2002) there has become an increased need for studies addressing the effect of such viral vectors, both on phenotype and function of the transduced cells. Adenoviral vectors are being used in several trials in both animal models and humans. A variety of strategies are being investigated, for example to target therapeutic genes to cells lacking them (Raper et al., 1998;Raper et al., 2002), to target suicide and toxic products specifically to cancer cells using modified vectors (Breidenbach et al., 2005;Kanerva et al., 2004), for use in vaccination strategies against HIV-1 (Barouch and Nabel, 2005) and to transfer tumour associated antigens to components of the immune system to stimulate a systemic specific immune response against tumour cells (Chen et al., 1996). Due to their pivotal role in the immune response, DCs have been targeted for gene transfer of these tumour associated antigens (Philip et al., 2000) with successful generation of tumour specific immune responses which can clear tumour cells on re challenge. From this body of work it appeared human DCs could be infected with adenoviral vectors and the transgene efficiently expressed. However, before this specific

system could be used verification of infection and protein expression was required (chapter 5) and the effects of the specific constructs used in this study needed to be investigated (chapter 6).

5.2 Specific materials and methods

5.2.1 Viral constructs

Recombinant adenoviruses containing the cDNA for MV proteins NP, F and H were kindly donated by Professor John Stephenson as discussed in section 2.7
Virus was amplified using standard protocols as outlined in section 2.7. After preparation of adenoviral stock, an estimate of viral infectivity was made by counting plaque-forming units (PFU) on 293 cells. This was done as outlined in chapter 2 by performing serial dilutions of the virus in duplicate then counting the one dilution that had approximately 50 to 100 plaques. These 50 plaques arose from 0.5ml of virus implying a titre of 100 x 1,000,000 (10^8) PFU per ml of virus stock. In the experiments shown, a PFU to cell ratio of 50:1 was used to infect cells. The endotoxin level in the virus preparations was also measured. This was $25pgml^{-1}$, which is less than reported levels in "endotoxin free" reagents (data not shown). This was to ensure any effect observed on DCs was due to infection and not LPS stimulation.

5.2.2 Short term line culture

In this study human monocyte derived DCs were infected at the immature state and then matured before use in stimulation assays. We did not infect mature DCs (mDC), since previous reports have suggested that mDCs are less readily

transduced due to low expression of the coxsackievirus and adenovirus receptor (CAR) (Bergelson et al., 1997;Rea et al., 1999).

5.2.2.1 Short term T cell lines stimulated with adenovirus infected DCs

T cell lines were grown using a previously described method (Leen et al., 2004a;Leen et al., 2004b). Briefly, monocyte derived DCs were cultured as outlined in section 2.6. These were then infected with adenovirus, either Rad35 (control), Rad68 (NP), Rad88 (H), or Rad95 (F) on day 5 at an MOI of 50 (these constructs are further described n chapter 6). After 24 hours cells were then matured using 100ngml^{-1} each of PGE_2, IL-6, human recombinant (hr)TNFα (all from Sigma UK) and hrIL-1β (Peprotech UK) for a further 24 hours. DCs were then counted and plated at a ratio of 1:10 (DC: T cell) with 2 x 10^6 autologous T cells in a 24 well plate in RPMI supplemented with 45% clicks medium (GIBCO) and 10% FCS. After 10 days the cells were harvested and re-plated at 2 x 10^6 cells per well in a 10:1 ratio of cell line to newly infected and matured autologous DCs. Half the media was changed every 3 days until day 18 when cells were harvested and re-plated in medium containing 50Uml^{-1} of human recombinant IL-2 (Roche) (figure 5.2).

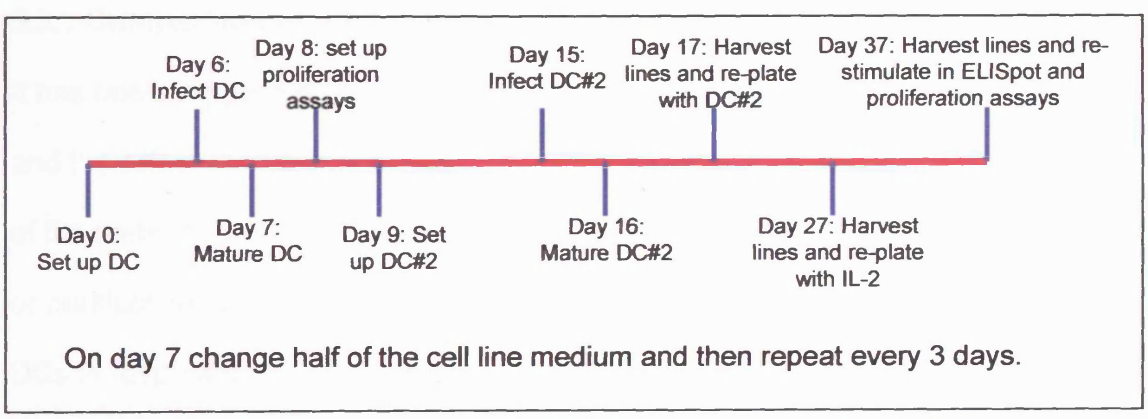


Figure 5.2 Schematic diagram of protocol for the production of T cell lines using autologous DCs infected with adenoviral constructs.

5.2.2.2 Re stimulation of T cell lines for proliferation assays

After a further 7-10 days in IL-2 the T cell lines were harvested and pooled, stained with CFSE, spun and re-suspended in fresh RPMI 10% FCS in half their original volume. The lines were then re-plated using 100μl per well and were co-cultured for 5 days with either autologous PBMC infected for 24 hours with the same Rad, or Rad35 or uninfected PBMC. After 5 days cells were harvested and stained with anti CD3 as previously described and analysed for proliferation by flow cytometry.

5.2.2.3 Re-stimulation of lines in the ELISpot assay

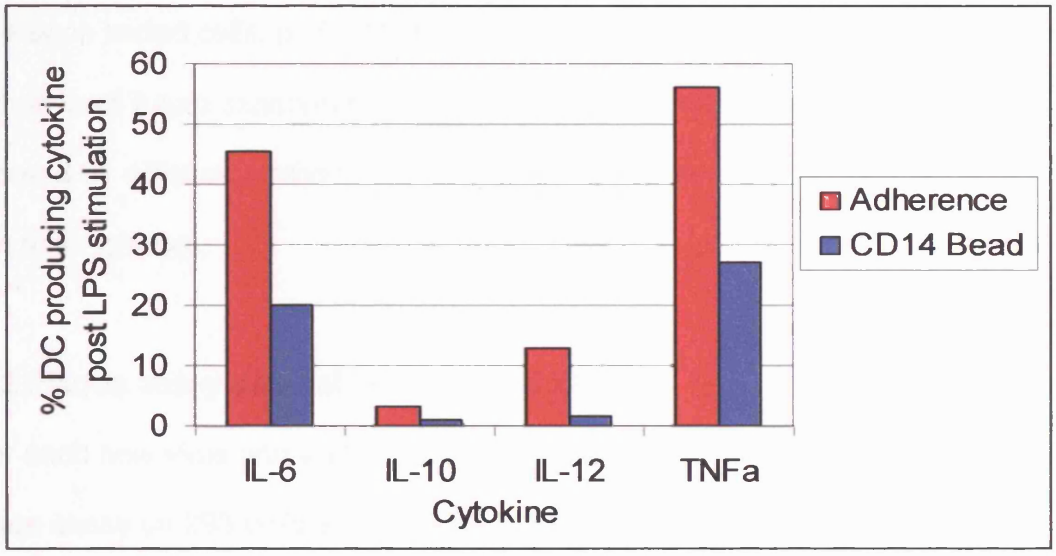
After a further 7-10 days in IL-2 the T cell lines were harvested and pooled, counted and plated in triplicate ELISpot wells at an optimized number of 10,000 cells per well in a 1:1 ratio with autologous PBMC that had been previously infected for 24 hours with the same Rad, control Rad or left uninfected. Peptide pools were used as previously described in chapter 3 with 40,000 cells per well of each line.

5.3 Results

5.3.1 Comparison of monocyte derived DCs prepared by different methods

It has been suggested that the phenotype, as detected by surface marker staining and the effect of maturation stimuli on these surface markers, is similar with either of the methods of DC preparation described in section 2.6.1, adherence to plastic or purification on magnetic beads. However when cytokine production by myeloid DCs in response to LPS stimulation was measured, as shown in figure 5.3 for 2 donors, the proportion of DCs producing cytokine was consistently less in DCs prepared with magnetic bead sorting than those prepared using adherence.

a)



b)

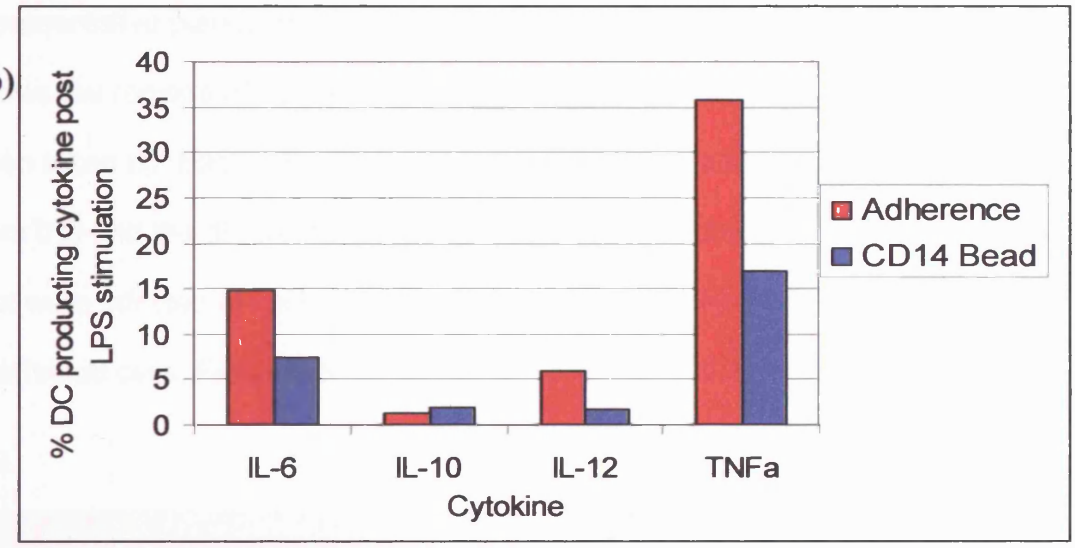


Figure 5.3: Comparison of cytokine production upon LPS stimulation in myeloid DCs prepared via adherence (red bars) or CD14 bead sorting (blue bars) in two control healthy donors, AW5 (a) and AW75 (b). Myeloid DCs were stimulated with 100ng/ml LPS for 18hrs with Brefeldin A added for the final 16 hours. Cytokine production was analysed by intracellular staining and flow cytometry data are expressed as the percentage of DCs producing cytokine.

There was a reduction in the percentage of DCs producing cytokine for all 4 of the cytokines tested, IL-6, IL-10, IL-12 and TNFα. The significance of this result was tested using the Wilcoxon paired rank statistical test and despite the small sample size, these differences were significant, as a decrease in total number of cells

producing all of the four cytokines in CD14 magnetic bead sorted cells compared to adherence sorted cells, $p < 0.015$. It was decided to continue to use the adherence method in all future experiments but take into consideration the fact that DCs prepared by different methods are not entirely the same, when comparing data from other groups.

5.3.2 Plaque assay determination of viral titre

After each new virus preparation an assessment was made of the titre by using a plaque assay on 293 cells as outlined in section 2.7.1.3. Figure 5.4 shows a representative plaque assay after infection of 293 cells with Rad35, where 5.4a) shows the regions of cell death due to viral replication where the red dye has not been taken up. Each one of these regions represents a single viral particle and from this and the dilution factor the viral titre could be calculated. 5.4b) shows cells that were infected at such a high concentration that they all died and c) shows uninfected cells. Figure 5.5 shows the same as figure 5.4 using light microscopy.

a) b) c)

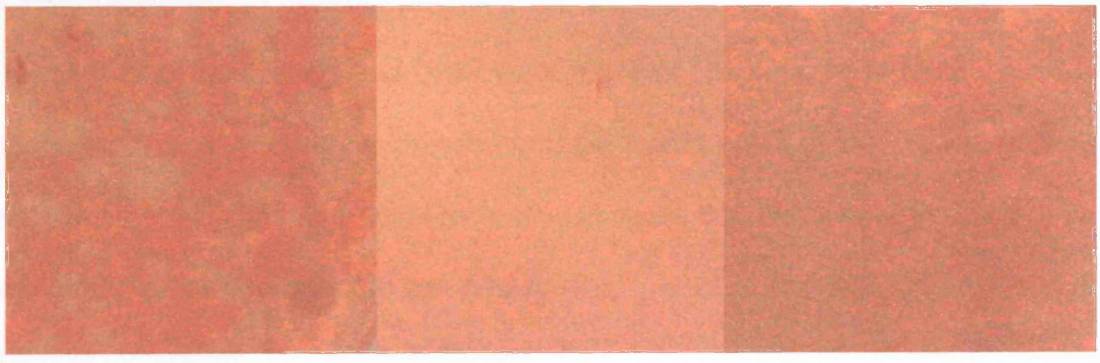


Figure 5.4: Photographs of plaque assays for virus stock PFU determination. a) 10^8 dilution where plaques are clearly visible, b) 10^6 dilution where all cells have been killed by the virus so have not taken up the dye and c) uninfected cells where all the cells are alive and therefore stained red by the neutral red dye.

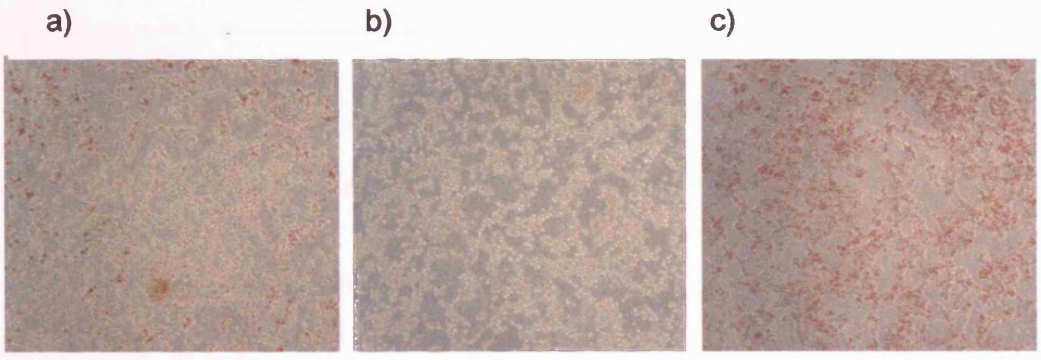


Figure 5.5: Light microscope photograph of plaque assays a) one plaque of virus killed 293 cells, b) 293 cells that have not taken up neutral red die as they have been killed by virus and c) uninfected 293 cells that have taken up the dye. (X10 x40 magnification).

5.3.3 Detection of βgal protein

The control construct Rad35 (containing cDNA coding for the β-galactosidase protein), was analysed for its capacity to infect certain cell types. This was done by detection of the β-galactosidase (β-gal) protein with the FDG assay (section 2.7.2.1). This was performed to verify that the construct could infect both human myeloid cell lines and various populations of human PBMC and DC.

5.3.3.1 Detection in monocytic cell lines Thp1 and U937

Monocytic cell lines, Thp1 and U937 were used for optimization of methods. The βgal protein was efficiently expressed and detected in both of the monocyte like cell lines tested, Thp1 and U937 as shown in figure 5.6. Cells were infected at an MOI of 50 for 24 hours before being harvested, pulsed with FDG and analysed on the flow cytometer.

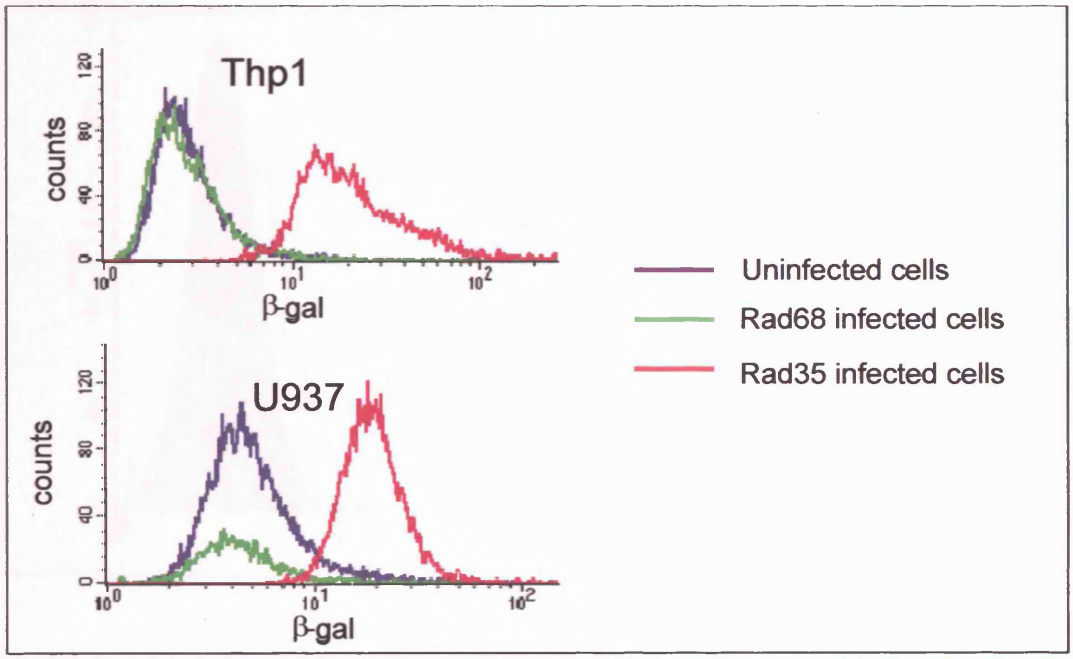


Figure 5.6: Expression of β-galactosidase protein in human myeloid cell lines. Thp1 and U937 cell lines were infected with Rad35 adenoviral construct (containing β-galactosidase cDNA) at an MOI of 50 for 24hours. They were then subjected to the FDG assay to detect β-gal protein by flow cytometry. Where the FDG substrate is forced into the cells by osmotic pressure and cleaved to a fluorescent product.

5.3.3.2 Time course of protein expression

A time course experiment, measuring βgal protein expression after 24 48 and 72 hours, was performed on the monocytic cell lines to find the time point with optimal protein expression. As shown in figure 5.7, after 24 hours expression in U937 cells, did not increase significantly. Therefore all assays were thereafter performed at this time.

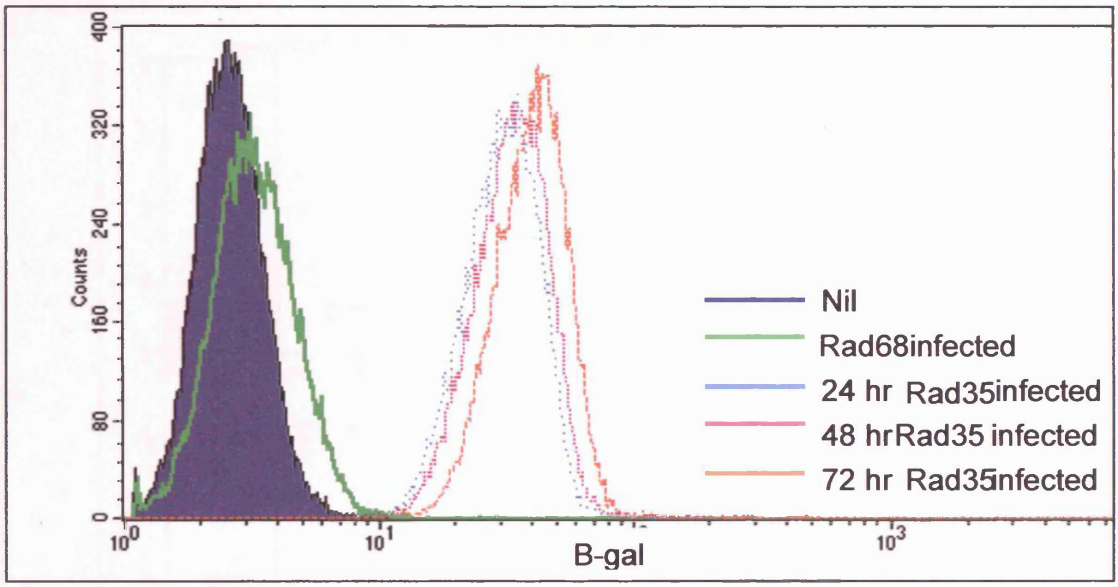


Figure 5.7 Time course of βgal protein expression. U937 cells were infected with Rad35 adenoviral construct (containing β-galactosidase cDNA) at an MOI of 50 for 24, 48 and 72 hours. They were then subjected to the FDG assay to detect β-gal protein by flow cytometry. Where the FDG substrate is forced into the cells by osmotic pressure and cleaved to a fluorescent product.

5.3.3.3 Protein expression in DC

As shown in figure 5.8 βgal was efficiently expressed in DCs after infection with the Rad35 adenoviral construct. This experiment was repeated in 5 different donors, using monocyte derived immature DCs infected at an MOI of 50 on day 6 of generation in GMCSF and IL-4. Propidium Iodide (PI), which enters dead cells, but not live cells, if they have not been fixed, was also added to all DCs including immature and LPS stimulated cells to ensure the constructs were not having a cytopathic effect on the DCs. As can be seen in figure 5.8 there was no more death in Rad35 infected cells than non-infected. However it was observed that death was higher in cells that had undergone the FDG assay compared to standard flow cytometry methods at levels of 25% compared to 15% (data not shown).

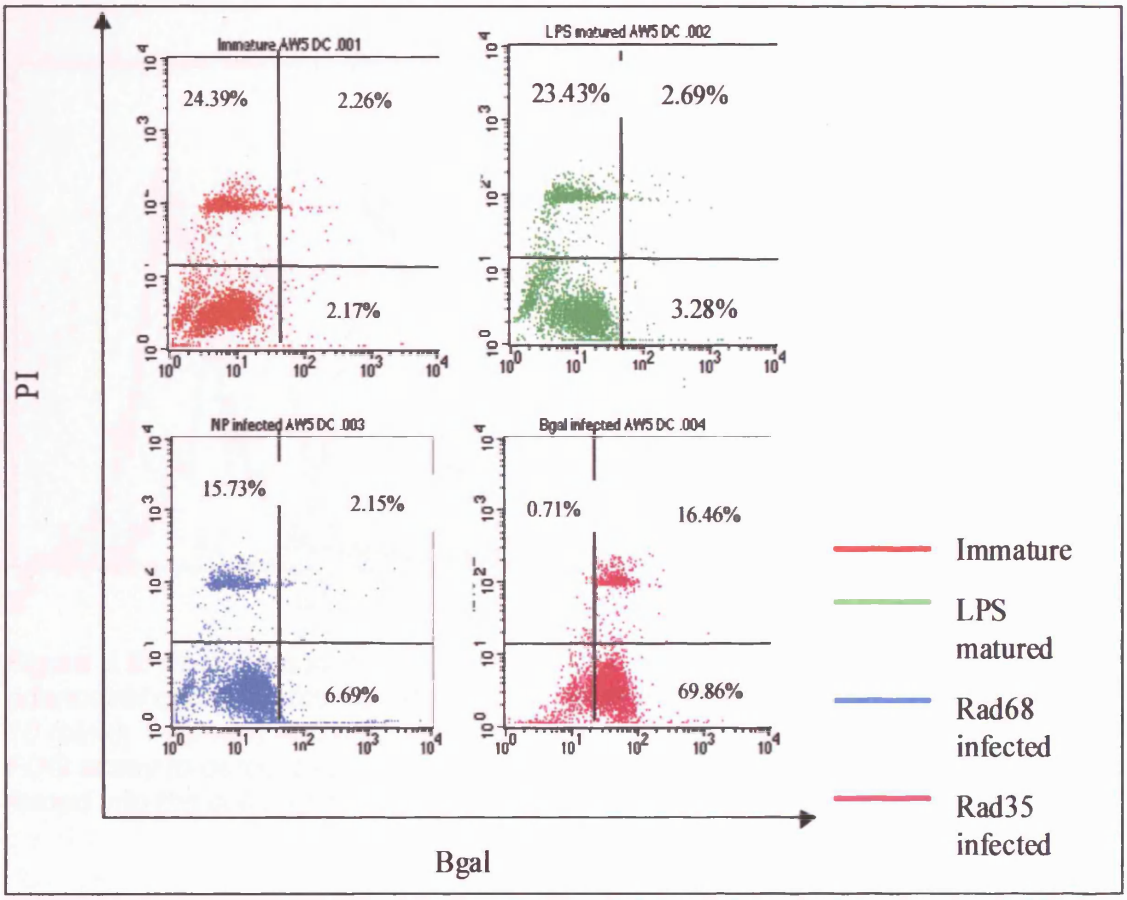


Figure 5.8: βgal protein expression in monocyte derived DCs and PI staining of dead cells, after infection with Rad35 (pink)adenoviral construct (containing β-galactosidase cDNA) or Rad68(blue) (containing MV NP cDNA) at an MOI of 50 for 24hours, immature (red) or LPS matured DCs (green). They were then subjected to the FDG assay to detect β-gal protein by flow cytometry. Where the FDG substrate is forced into the cells by osmotic pressure and cleaved to a fluorescent product.

5.3.3.4 PFU titration in DC

A titration was done of Plaque forming units (PFU) of virus per DC (multiplicity of infection –MOI) using the Rad35 adenoviral construct. The result in figure 5.9 shows that infection does titrate out, with βgal protein no longer detectable at a ratio of 1 PFU per DC.

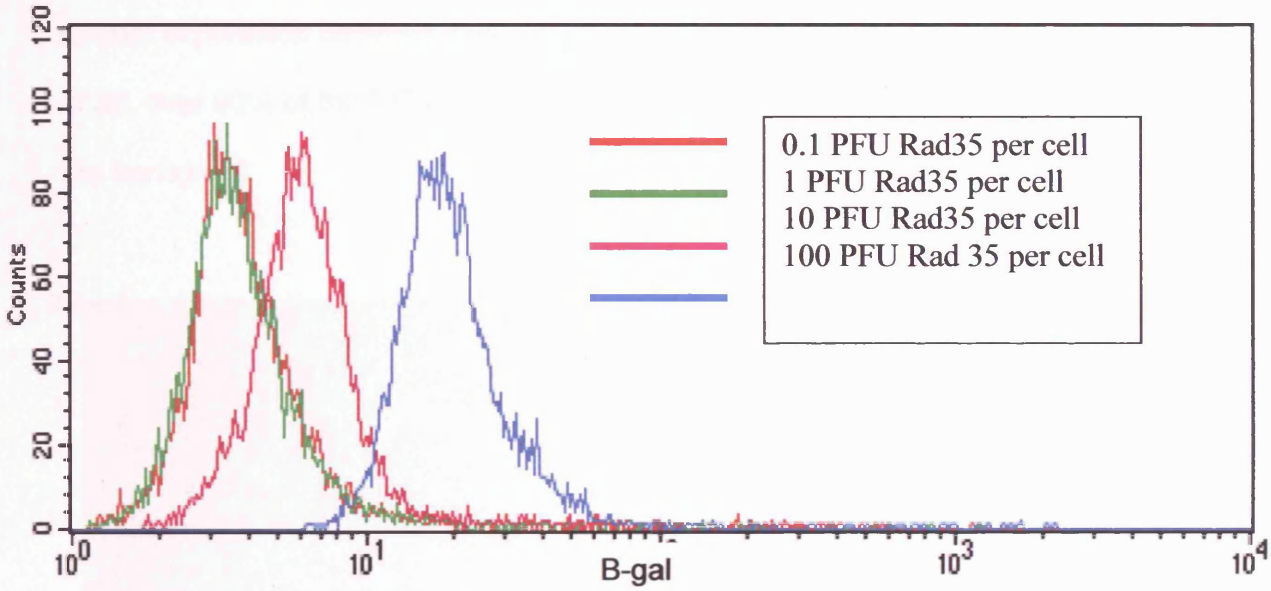


Figure 5.9: PFU titration in DCs. Monocyte derived DCs were infected with Rad35 adenoviral construct (containing β-galactosidase cDNA) at an MOI of 100 (blue), 10 (pink), 1 (green) and 0.1(red) for 24 hours. They were then subjected to the FDG assay to detect β-gal protein by flow cytometry. Where the FDG substrate is forced into the cells by osmotic pressure and cleaved to a fluorescent product.

5.3.3.5 Visual verification of FDG staining using X-gal

To verify the results of β-gal protein staining seen with the FDG substrate, Rad35 infected DCs were also stained with 5-Bromo-4-Chloro-3-indolyl β-D-Galactopyronoside (X-gal) as outlined in section 2.7.2.3. Figure 5.10 shows DCs that have either been infected with Rad35 and contain β-galactosidase (5.10a) or DCs that have been infected with Rad68 and do not contain the β-galactosidase enzyme (5.10b) and then stained with X-gal. The DCs have then been pictured under a light microscope and as can be seen, there is blue staining in the cells that contained the enzyme, verifying the FDG staining data. Interestingly there appears to be some cells that stain heavily and are very blue but the majority of the other DCs also stain blue but to a lesser extent, compared with no staining at all in the

DCs that were infected with Rad68. This result suggested a variable level of β-gal protein expression between individual cells. This verified the finding that at an MOI of 50, over 90% of the DC were infected with the constructs and efficiently express the transgene.

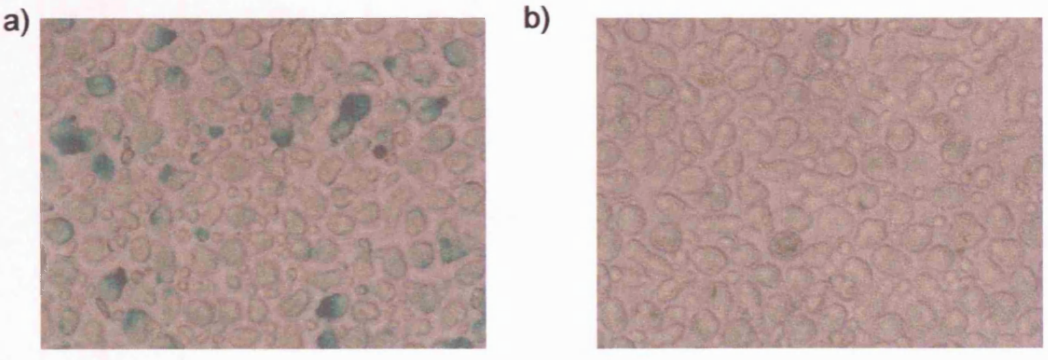


Figure 5.10: X-gal staining of β-gal protein in Rad35 infected DCs. Monocyte derived DCs were infected with Rad35 adenoviral construct (containing β-galactosidase cDNA) at an MOI of 50 for 24 hours, before being harvested and stained with X-gal for a further 24 hours before visualisation on a light microscope (x10x40) a) Population of Rad35 infected DCs stained with X-gal, b) Population of Rad68 infected cells stained with X-gal DC.

5.3.3.6 Tropism of virus to sub sets of PBMC

It has been suggested that due to varying levels of the coxsackie and adenovirus receptor (CAR) on the surface of different cellular subsets that the virus may have a tropism to one subset of PBMC over others. Since this study used PBMC which had been infected with virus, as feeder cells to re-stimulate cell lines that had been grown to adenovirus infected DCs, it was important to investigate the tropism of these adenoviral constructs in this system. Figure 5.11 shows PBMC that were infected at an MOI of 50 for 24hours with Rad35 then harvested and treated with FDG to detect β-gal protein. The infected PBMC were stained for lineage markers CD3, CD8 and CD4 (T cells), CD19 (B cells) and CD14 (monocytes). As shown in

figure 5.11, all of the cell subsets stained equally well. This was also true at lower MOI where a smaller percent of each subset was infected but there was no tropism for one sub set over another (data not shown).

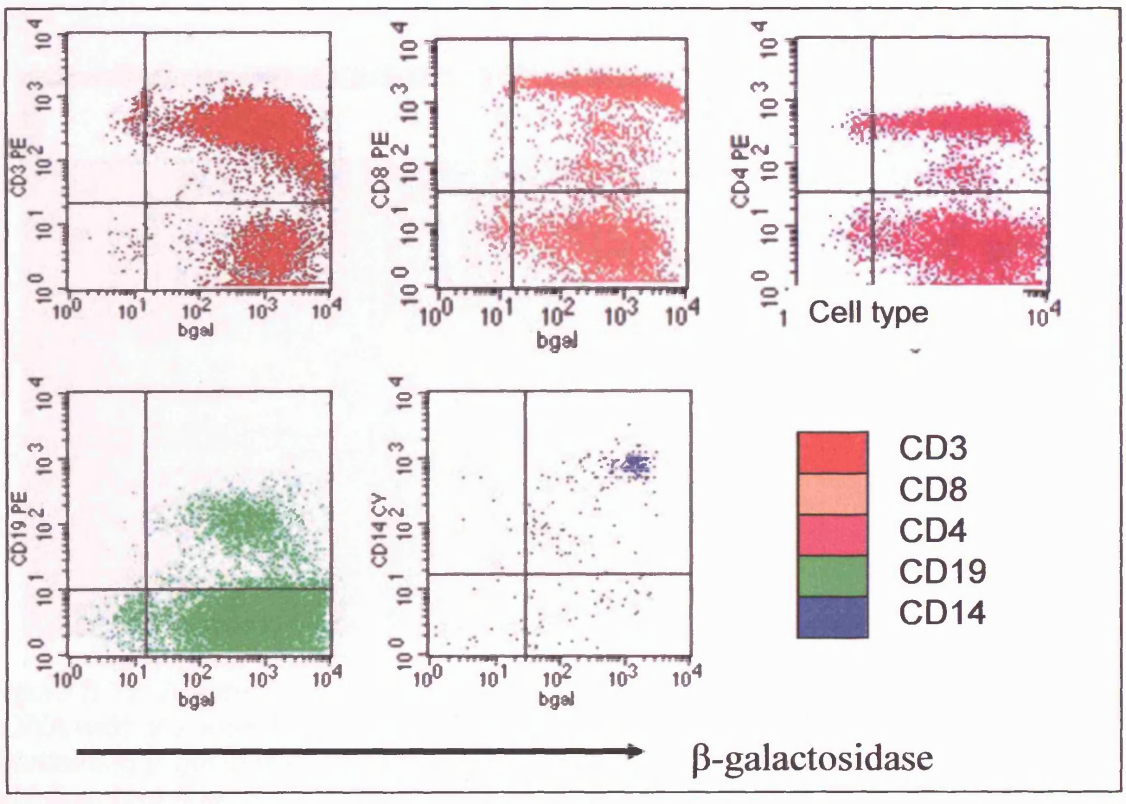


Figure 5.11: Flow cytometry showing with FDG staining that the adenoviral vector has no tropism for a specific cell subset. Human PBMC were infected with Rad35 adenoviral construct (containing β-galactosidase cDNA) at an MOI of 50 for 24 hours. The cells were then analysed using flow cytometry all cells were gated using a live cell gate and then for CD14 (blue) analysis, a monocyte gate was used. The other markers were analysed on cells from within the lymphocyte gate. These gates are based of side and forward scatter properties of the PBMC.

5.3.4 Detection of measles NP protein after infection with adenoviral constructs

5.3.4.1 Polymerase chain reaction -PCR of the NP cDNA in Rad68 constructs

In order to ensure that the construct contained the correct cDNA, PCR (as outlined in section 2.7.2.5) was performed using primers specific for the 5' and 3' ends of

the NP cDNA and the adenovirus preparation was used as a template. The results of this PCR showed that the construct does contain the correct cDNA, the NP cDNA is a size of 1.5Kb and this is where the band is detected (figure 5.12). No band was detected in control reaction using Rad35 as the template.

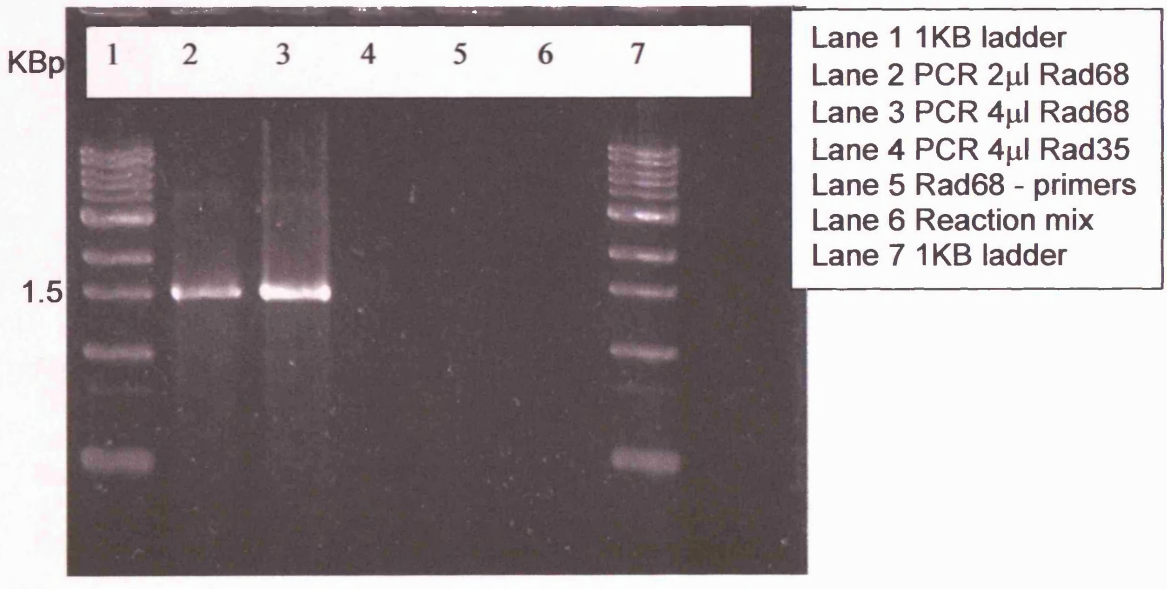


Figure 5.12: Agarose gel electrophoresis of PCR products using primers for MVNP cDNA with adenoviral constructs Rad68 (containing NP cDNA) and Rad35 (containing β-gal cDNA) as templates, run on a 1.5% agarose gel and stained with ethidium bromide.

5.3.4.2 Detection of MV NP protein in monocytic cell lines, Thp1 and U937

Once the construct was verified, several different antibodies (see table 2.3) were used to stain cells by flow cytometric analysis with intracellular staining, to detect the NP protein in cells infected with Rad68. Although successful in the monocytic cell lines (figure 5.13), this was not successful in the human DCs (figure 5.14) due to non-specific binding of the anti measles polyclonal serum used. Therefore other methods of MV NP protein detection were used, as outlined in section 5.3.4.3 onwards.

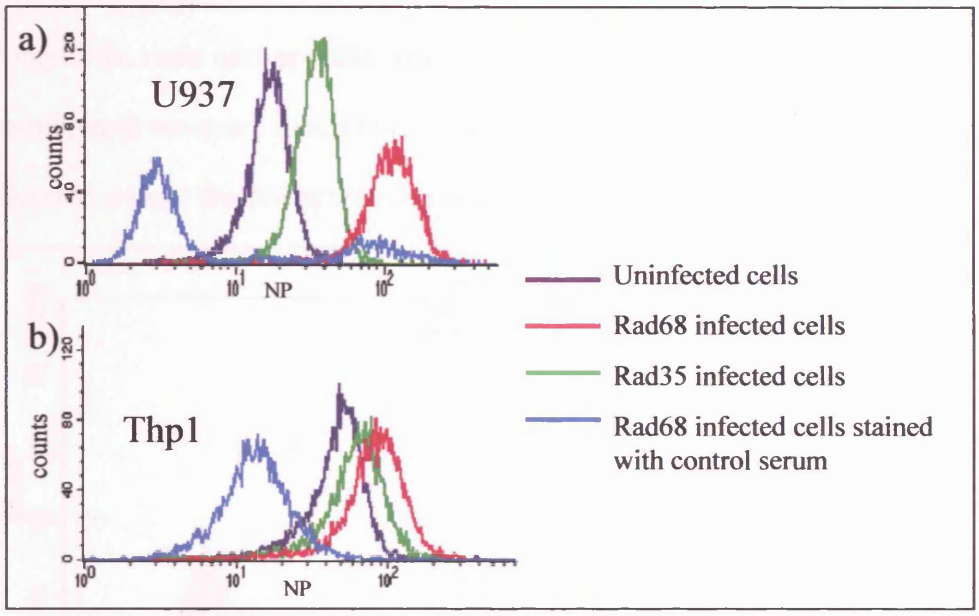


Figure 5.13 Staining of cell lines with anti measles rabbit serum. U937(a)) and Thp1(b)) monocytic cell lines were infected with Rad35 (green), Rad68 (pink) containing MV NP cDNA or left uninfected (purple) for 24 hours. The cells were then stained with anti MV rabbit serum or control rabbit serum (blue) at a dilution of 1/100 and then a second layer antibody anti rabbit PE before being analysed by flow cytometry.

Cell lines Thp1 and U937 were infected with the Rad68 construct (containing MV NP full length cDNA) and then stained with anti measles rabbit serum (kindly provided by Dr Kenth Gustaffson, ICH, UCL) at a dilution of 1/100, 24 hours post infection. Figure 5.13 appears to show the detection of NP in cell lines infected with Rad68. However some non-specific binding was also demonstrated with the increased staining of Rad35 infected cells (green line). This experiment used polyclonal rabbit serum however this result could not be reproduced with the anti measles mouse and human sera, again due to non-specific binding despite efforts to block Fc binding sites (data not shown).

5.3.4.3 Detection of MV NP protein in Rad68 infected DC

On dendritic cells non-specific binding of all anti measles sera tested was seen (for example anti measles rabbit serum shown figure 5.14). This made it technically difficult to detect the NP protein by flow cytometric analysis.

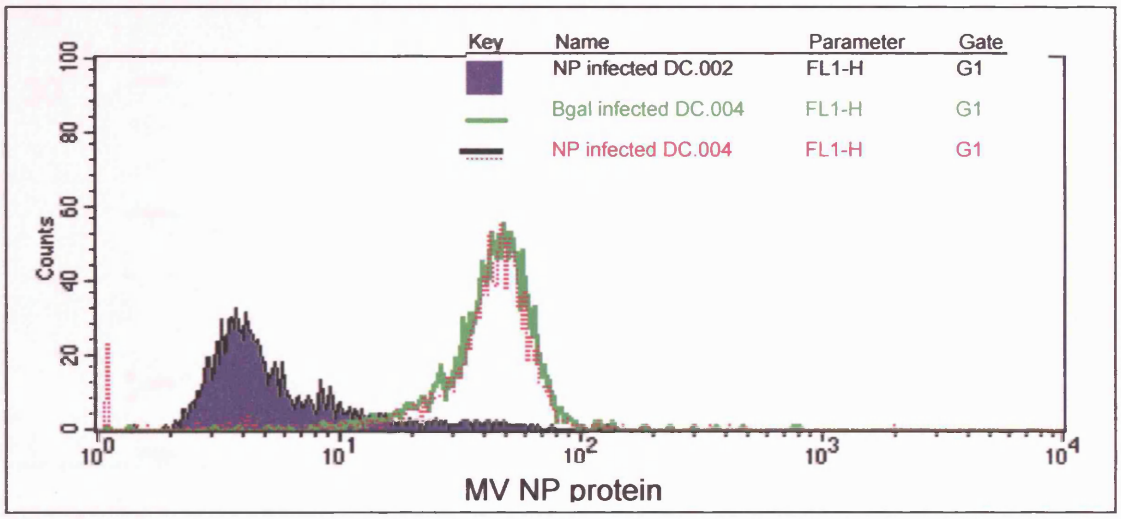
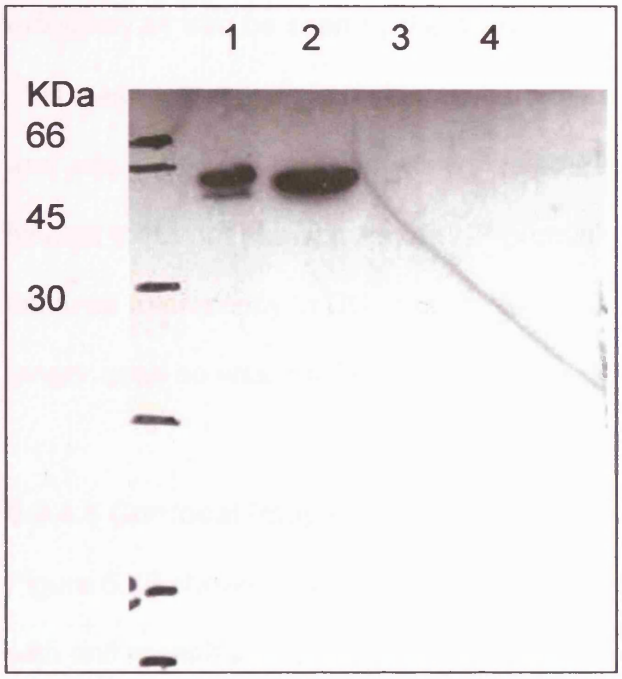


Figure 5.14: Staining of Rad68 infected DCs with anti MV serum. Rad68 –MV NP construct (pink) and Rad35 – β-gal construct (green) infected DCs were infected for 24 hours then stained with anti measles rabbit serum compared to control serum (filled histogram)and analysed using flow cytometry.

5.3.4.4 Western blotting to detect MV proteins

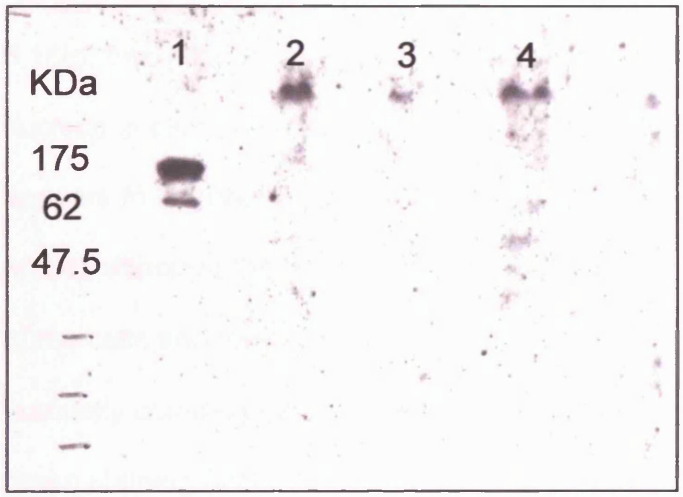
Using the more sensitive technique of western blotting, infected DC cell lysates were probed using all the sera available, each raised to either whole MV or NP protein. The 60KDa NP protein could be detected in Rad68 infected 293 cells compared to Rad35 infected 293 cells (figure 5.15a) However, as shown in figure 5.15b), for human anti measles serum, despite low background, only the positive control could be detected on this gel. This positive control was a commercially obtained cell lysate from measles infected Vero cells.

a)



Lane 1 Vero cell lysate
Lane 2 Rad68 293 cell lysate
Lane 3 Rad35 293 cell lysate
Lane 4 uninfected 293 cell lysate

b)



Lane 1 Vero cell lysate
Lane 2 Rad68 DC lysate
Lane 3 Rad35 DC lysate
Lane 4 uninfected DC lysate

Figure 5.15 Western blots to detect MV NP using human polyclonal serum in a)293 cells and b) human DCs. The NP protein is 60KDa. Adenoviral construct infected cells were harvested 24 hours post infection, cell pellets were re-suspended in lysis buffer and used for western blot analysis. Positive control well contained MV infected Vero cell lysate treated in the same way. Blots were probed with anti MV human serum from an SSPE patient kindly provided by Prof John Stephenson.

It appears that the proteins extracted from the DCs do not run through the gel efficiently as can be seen by the accumulation of staining remaining in the wells. This was a reproducible problem when trying to detect proteins in the DC lysates and was confirmed by coomassie blue staining of gels run in parallel from DC cell lysates (data not shown). As the NP protein was then detected by different confocal microscopy in DC (see section 5.3.4.5) this method of detection was no longer used so was not further optimized.

5.3.4.5 Confocal imaging of MV NP protein in adenoviral infected DC

Figure 5.16 shows DCs that were infected with Rad68 and after 24 hours, stained with anti measles serum a), and human serum isotype control b). Also DCs infected with Rad35 and stained with anti measles serum are shown in figure 5.16c). The measles protein is stained green, the actin cytoskeleton in red and the nucleus in blue. Figure 5.16d) shows the morphology of a single DC. There appears to be more staining in figure 5.16a) than in the two control figures (5.16b) and c), although the number of cells showing strong staining is a small proportion of the cells and fewer that were seen with the βgal in the FDG assay. When manually counting each field 71% of the Rad68 infected cells were found to show green staining (indicating NP protein expression) where as only 13% of the cells infected with Rad35 are stained green. This is shown more clearly in figure 5.16e) and f) which are the pictures from 5.16 a) and c) without the other staining colours, therefore only showing staining for NP.

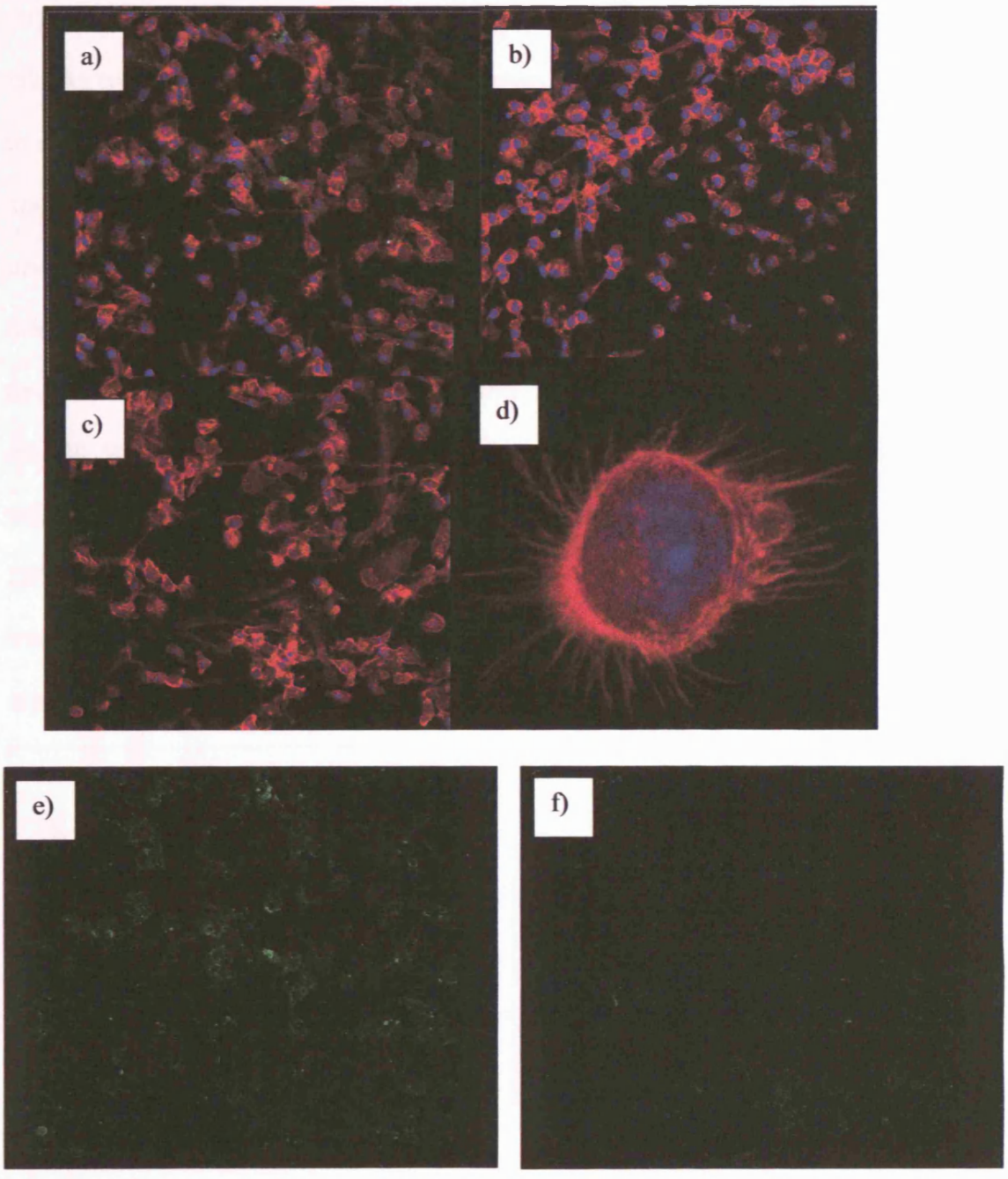


Figure 5.15: Confocal images showing DC morphology and staining for measles NP (green) actin cytoskeleton (red) and nucleus (blue) in a) Rad68 and c) Rad35 infected DC stained with anti measles human serum, b) Rad68 infected cells stained with control serum and d) high power image of one DC. Confocal staining of e) Rad68 infected DCs and f) Rad35 infected DCs stained with anti measles human serum showing only the expression of the MV N protein in human DC without actin cytoskeleton and nucleus.

5.3.5 T cell lines grown to autologous Rad infected DC

Once verification of the methodology had occurred and the gene products of cDNAs within the adenoviral constructs were detected in DCs, it was then possible to use these DCs to stimulate autologous T cells in order to culture T cell lines specific for the part of the MV protein which had been most dominantly processed and presented by the DCs. Firstly, to establish whether T cells proliferated to DCs infected with adenoviral vectors containing measles proteins, DCs were prepared from 3 healthy MV immune volunteers and were infected on day 6 with Rad35, Rad68, Rad88 or Rad95 adenovirus vector, half of the cells were then matured with the cocktail of cytokines TNFα, IL-1β, IL-6 and prostaglandin E_2 (Leen et al., 2004a). The other half of the DCs were kept immature. Autologous PBMC were then stained with CFSE and co-cultured in a 10:1 ratio with the DCs for 6 days and analysed by flow cytometry. Figure 5.17 shows 1 representitive of 3 experiments.

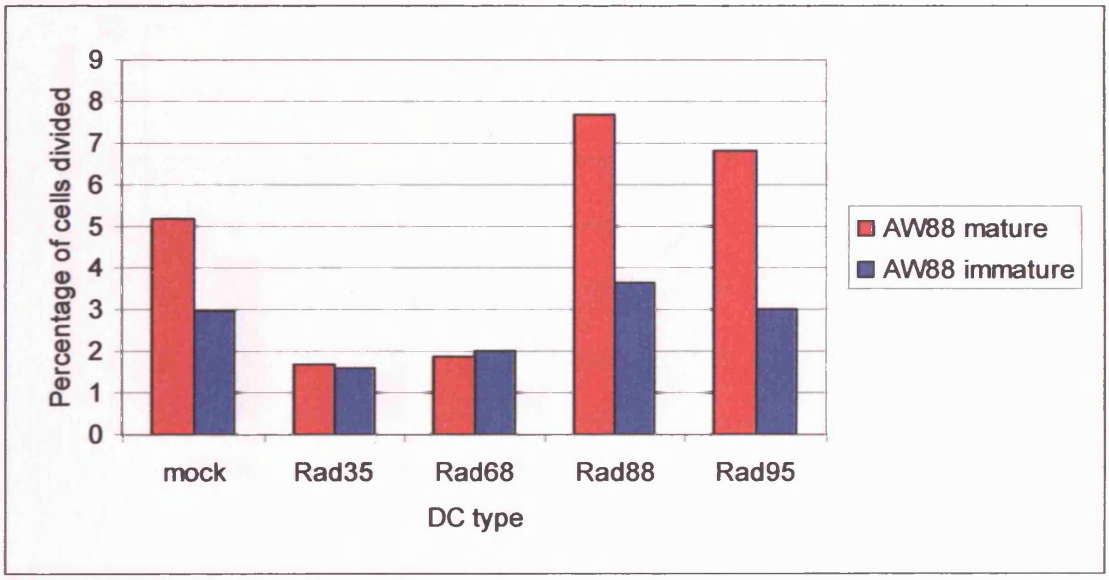


Figure 5.17: Percentage proliferation of non adherent AW88 autologous PBMC to adenovirus infected immature (blue bars) or mature (red bars) DCs either uninfected, containing β-gal protein (Rad35) or containing MV proteins NP (Rad68), H (Rad88) and F (Rad95). As measured by percentage of CD3+cells dividing as determined by CFSE staining as analysed using flow cytometry.

T cells did not appear to proliferate well to the NP protein. However T cells did proliferate to F and H proteins when presented by mature DCs. Therefore autologous, matured DCs were used to stimulate MMR02 T cells from different time points post vaccination in order to grow out short term T cell lines. Prior to this, proliferation of MMR02 PBMC from pre (A) and 10 days post (C) MMR vaccination was tested to the different Rad infected DCs in the same way. All the DCs were matured for the experiment shown in figure 5.18. Figure 5.18 shows that proliferation increases post vaccination especially to MV F (Rad95) and H (Rad88) proteins. Interestingly, where the proliferative response does not increase either on maturation of the DCs (figure 5.17) or post vaccination (figure 5.18), for instance to Rad35 and Rad68 constructs, the proliferation to infected DCs is in fact less than the proliferation to uninfected DCs. This will be further discussed in chapter 6.

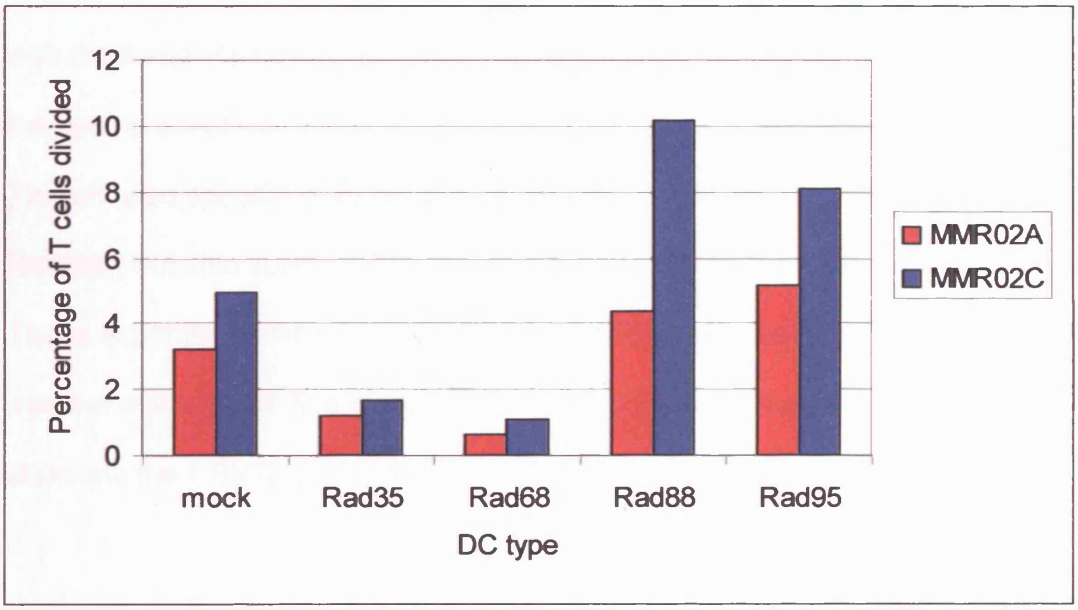


Figure 5.18: Proliferation of MMR02 PBMC from pre and post MMR vaccination to DCs infected with Rad containing MV proteins. Percentage proliferation of MMR02 PBMC from pre (A) (red bars) or 10 days post (C)(blue bars)MMR vaccination, to mature DCs either uninfected, containing β-gal (Rad35) or containing MV proteins NP (Rad68), H (Rad88) and F (Rad95). As measured by percentage of CD3+cells divided after 6 days of culture by CFSE staining, analysed using flow cytometry.

5.3.5.1 Re-stimulation of T cell lines grown to autologous Rad infected DCs with fresh autologous Rad infected DCs.

T cell lines from the vaccinated individual MMR02 were grown according to the protocol outlined in section 5.2.5.2. It was observed in preliminary experiments that prior to re-stimulation with fresh Rad infected autologous DCs for analysis of responses, T cell lines must be allowed to recover in IL-2 for 10 days to achieve maximum proliferation and cytokine production (data not shown). T cell lines were grown to Rad infected DCs from MMR02. The T cell lines grown were labelled according to the Rad construct they had been stimulated with and the time point that the responder PBMC were taken from post vaccination (see figure 4.1), e.g. Rad68B was a line grown to autologous DCs infected with Rad68 with PBMC taken from time point B. Figure 5.19 shows the number of cells producing IFNγ as assayed by ELISpot in response to autologous PBMC, that were either infected with the same Rad that was used to grow the line- in this case Rad68, infected with the control construct, Rad35 or uninfected and used as antigen presenting cells. This showed specific IFNγ production over background in the cell line MMR02, Rad68E, but little specific IFNγ release in line Rad68B from the same individual. These experiments were only done on one of the vaccinated volunteers as MMR01 was not available to give blood regularly enough to produce the DCs required to stimulate the PBMC to culture the T cell lines.

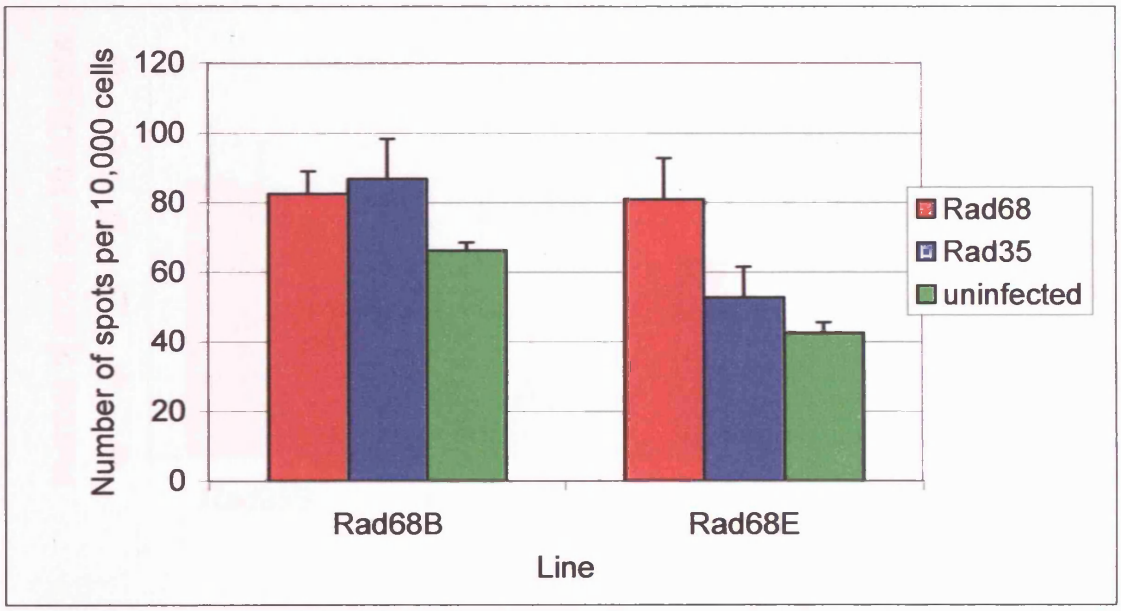


Figure 5.19: Response of 2 T cell lines grown from MMR02 PBMC from time points B and E, grown to DCs expressing the MV NP protein re-stimulated with autologous PBMC infected with Rad68 (red), Rad35 (blue) or uninfected (green). Number of cells in the cell line producing IFNγ per 10,000 was assayed by a standard ELISpot stimulated by PBMC in a 1:1 ratio. Values represent the mean and error bars, one standard deviation of triplicate wells.

Figures 5.20 and 5.21 show the results of re-stimulating the T cell lines from MMR02, grown to autologous DCs infected with Rad95 (figure 5.20) and Rad88 (figure 5.21). As in figure 5.19 the lines were again re-stimulated with autologous PBMC that had themselves been infected with the relevant or irrelevant Rad or left uninfected. None of these lines shown appeared to have a specific response to the measles virus proteins since all responded to Rad35 infected control PBMC as much as to the PBMC infected with the specific adenoviral construct. This could be due to T cells being stimulated by adenoviral proteins also present in Rad35 and so T cells being specific for the adenoviral vector proteins rather than the specific MV protein. However where there were enough of the cell lines left after the initial analysis, they were re-stimulated with peptide pools relevant for the protein the line was grown to as outlined in the next section.

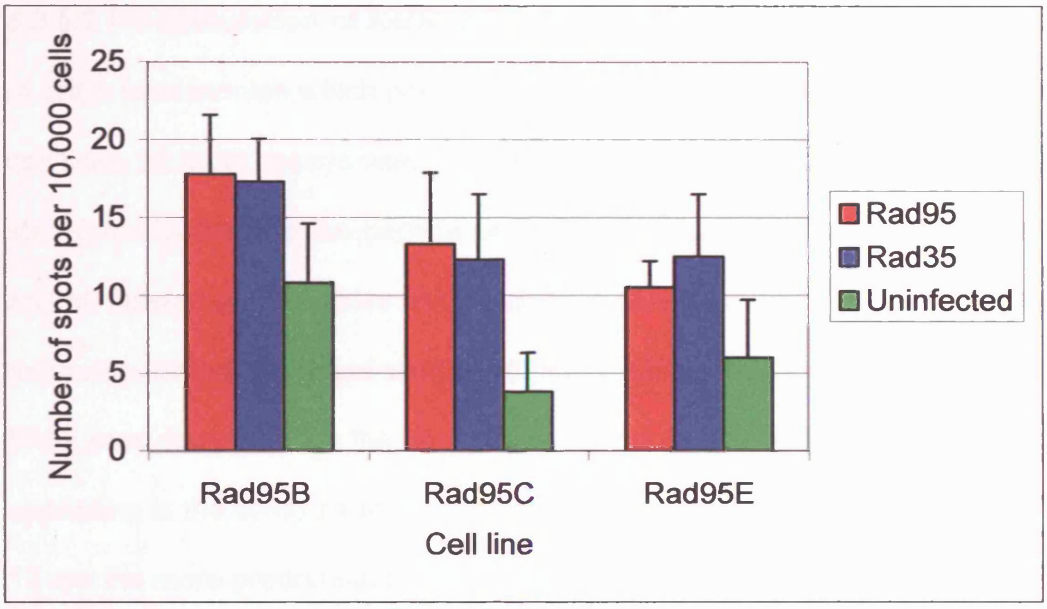


Figure 5.20: Response of 3 T cell lines grown from MMR02 PBMC from time points B, C and E, grown to DCs expressing the MV F protein re-stimulated with autologous PBMC infected with Rad95 (red), Rad35 (blue) or uninfected (green) in a 1:1 ratio. Number of cells in the cell line producing IFNγ per 10,000 was assayed by a standard ELISpot and results expressed as mean and 1 SD of triplicate wells.

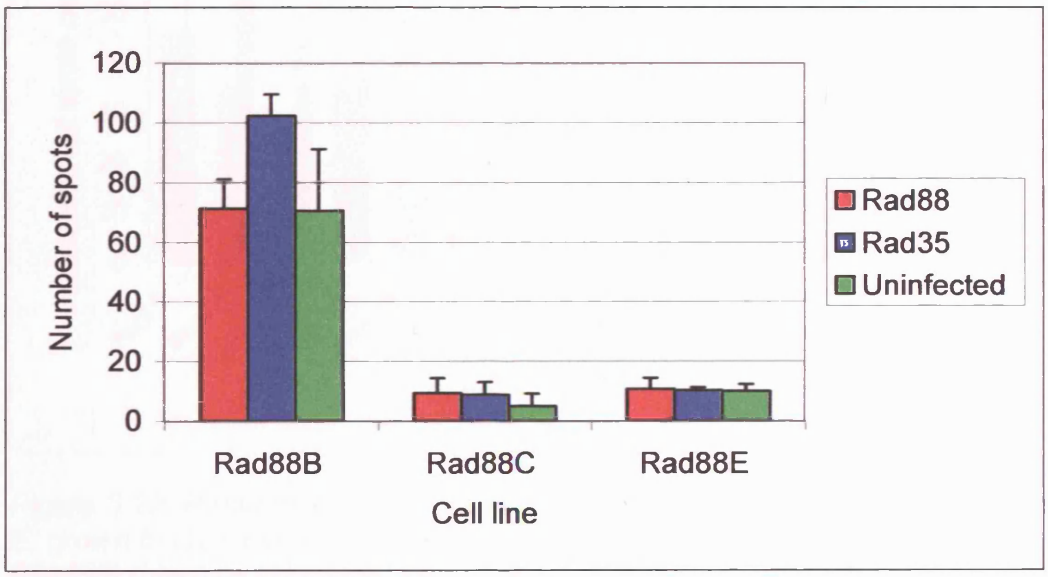


Figure 5.21: Response of 3 T cell lines grown from MMR02 PBMC from time points B, C and E, grown to DCs expressing the MV H protein re-stimulated with autologous PBMC infected with Rad95 (red), Rad35 (blue) or uninfected (green) in a 1:1 ratio. Number of cells in the cell line producing IFNγ per 10,000 was assayed by a standard ELISpot and results expressed as mean and 1 SD of triplicate wells.

5.3.5.2 Re-stimulation of MMR02 T cell lines with MV peptide pools

In order to determine which peptides in the MV proteins were recognised by the T cell lines, ELISpot assays were carried out with the T cell lines from MMR02 stimulated by the relevant peptide pools. Cells were plated at 40,000 cells per well and stimulated with peptides as described in chapter 3. Figure 5.22 shows the response of Rad35E (β-gal stimulated) and Rad95E (MV F stimulated) cell lines to MV F peptides. Although the background for the Rad95 line is high the pattern appearing is the same as for the normal volunteers, in that pools 8, 9, 10, 11 and 12 are the more predominant pools. However these are not statistically significant.

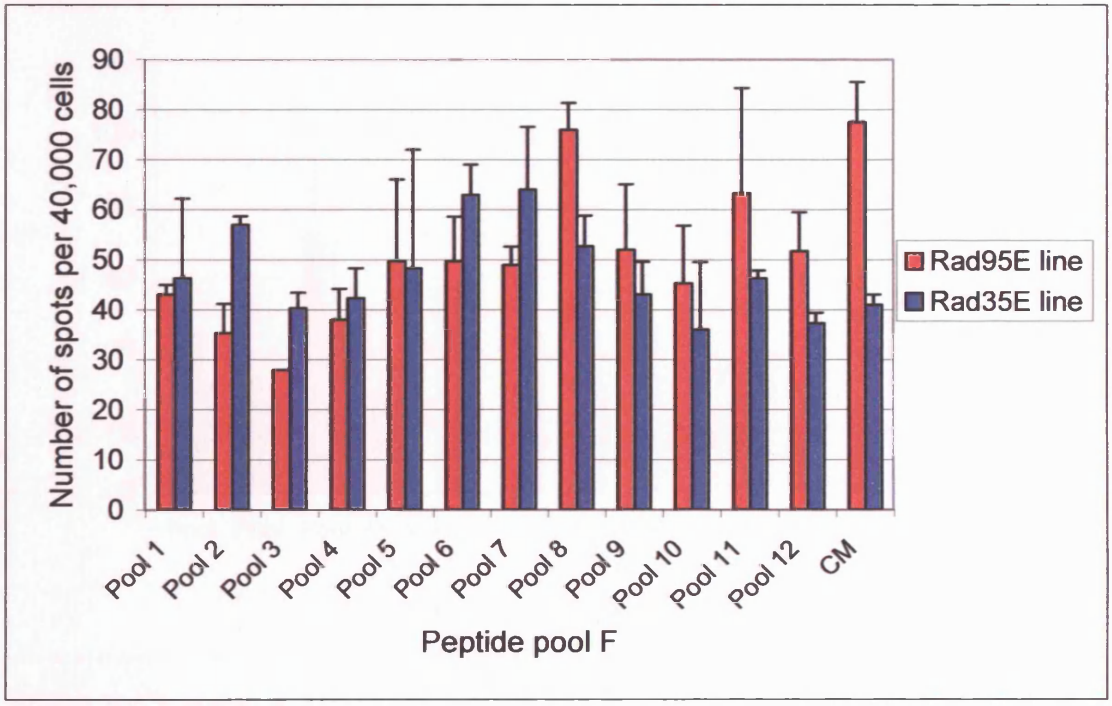


Figure 5.22: Response of 2 T cell lines grown from MMR02 PBMC from time point E, grown to DCs expressing the MV F protein (Rad95) (red) and the β-gal protein (Rad35) (blue) re-stimulated with the pools of peptides derived from the MV F protein. Number of cells in the cell line producing IFNγ per 40,000 was assayed by a standard ELISpot and results expressed as mean and 1 SD of triplicate wells.

Figure 5.23 shows the responses of T cell lines grown to Rad68 infected DCs (MV NP) or Rad35 infected DCs, grown from PBMC of MMR02 taken at time point C to

MV NP peptide pools, as measured by ELISpot. However no single pool of peptides seems to induce a response which would indicate a dominant epitope in any one of the pools that is statistically significant, although the response to NP peptide pools 3 and 6 may warrant further investigation if this experiment was to be repeated. There were not enough of the cell lines grown to Rad88 infected DCs (MV H protein) to re-stimulate these with the MV H peptide pools, nor were there enough of the cell lines left to re-stimulate the cells with MV peptides that had been separated from the highlighted MV peptide pools.

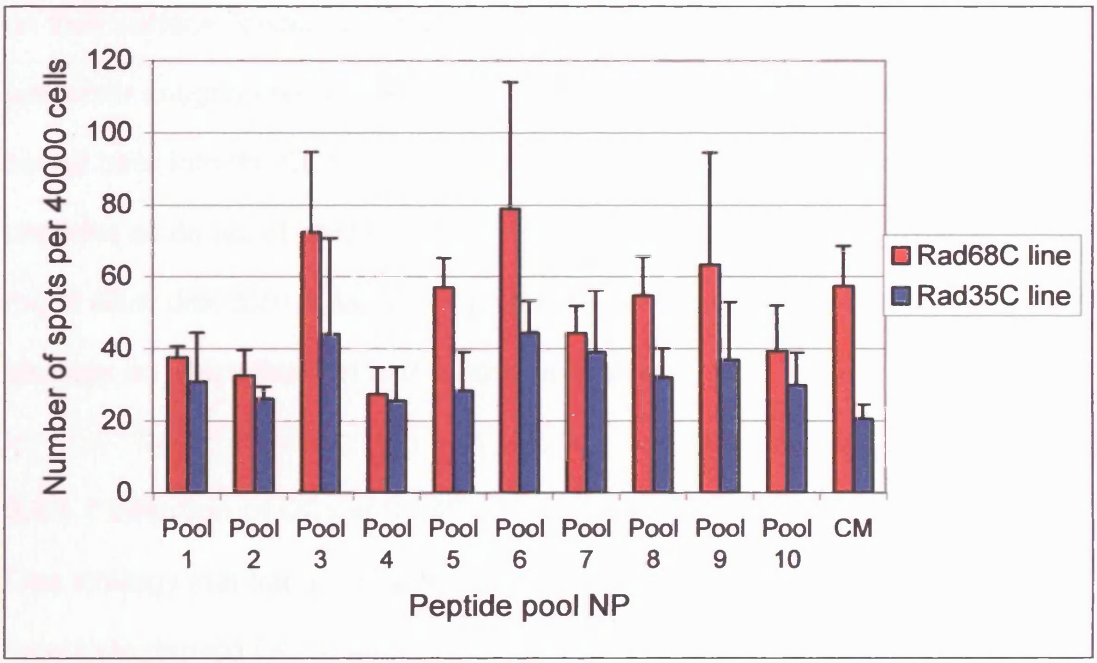


Figure 5.23: Response of 2 T cell lines grown from MMR02 PBMC from time point C, grown to DCs expressing the MV NP protein (Rad68) (red) and the β-gal protein (Rad35) (blue) re-stimulated with the pools of peptides derived from the MV NP protein. Number of cells in the cell line producing IFNγ per 40,000 was assayed by a standard ELISpot and results expressed as mean and 1 SD of triplicate wells.

5.4 Discussion

5.4.1 Alternative strategies for detecting epitopes

With the modified aim of identifying epitopes in adults and not quantifying the response in the first instance, alternative strategies to identify potential T cell epitopes were explored. The strategy identified, involved using dendritic cells as APC. However instead of pulsing these cells with MV peptides, they would be infected with antigen to more closely mirror the natural route of infection, and subsequent processing and presentation of antigen. These DCs could then be used as the stimulus to grow out short-term T cell lines to MV antigens presented on their surface. It was hypothesized that this might result in an amplification of the precursor antigen specific memory T cells. These specific T cell lines would then be put back into the ELISpot assay, and stimulated with pools of overlapping MV peptides as demonstrated by Goulder et al (Goulder et al., 2001). This strategy might allow detection of individual peptide sequences of importance. This was the strategy on which this part of the work was based.

5.4.1.1 Infection of DCs with MV

One strategy that had been previously discussed was that of using MV to infect monocyte derived DC cultures and use these to present antigen to T cells. However as discussed in section 1.3.5 and 1.3.8, there have been several reports of cytopathic and negative regulatory effects of MV infection on DCs. These effects include; Fas mediated apoptosis of DCs (Servet-Delprat et al., 2000a), Syncytium formation post activation of DCs via CD40 ligation and decreased capacity to produce IL-12 (Fugier-Vivier et al., 1997) and modulation of signalling via CD40 (Servet-Delprat et al., 2000b). There has also been evidence that the expression of

MV proteins F and H on the DC cell surface inhibit mitogen induced T cell proliferation by overcoming the positive co stimulation signals from the DC to the T cell (Klagge et al., 2000). Although it has also been reported that MV infection induced maturation and IL-12 production by immature DCs it was also observed that these DCs could not sustain proliferation of T cells to mitogen (Schnorr et al., 1997). Thus although several mechanisms may be implicated, it is well documented that MV infection of DCs can substantially decrease the proliferative capacity of activated T cells, therefore it was necessary to investigate another route of MV protein delivery to antigen presenting cells.

5.4.1.2 Replication deficient adenoviruses (Rad)

Despite the reported lack of the most categorised of adenovirus receptors, the CAR on the human DC surface, at an MOI of 50, up to 90% of cells were efficiently infected with the adenoviral constructs as shown by staining with coloured substrates for the reporter gene β-galactosidase. The measles proteins themselves were harder to detect, as levels of protein in the cells were probably low and required the amplification of the signal such as that which occurred with the reporter gene substrates. This theory was further strengthened by the failure to detect the reporter gene with an anti β-galactosidase antibody in human DCs (data not shown). However after using several different techniques it was possible to demonstrate expression of the NP protein in human DCs via confocal microscopy. Once verification of infection was achieved, work shown in this chapter went on to optimise the culture of T cell lines from PBMC obtained before and after MMR vaccination, stimulated by DCs infected with adenovirus containing MV proteins. Although some specific proliferation and production of IFNγ by the cell lines that

were grown to adenoviral infected DCs was observed, it remained difficult to observe a measles -specific response over the response to the control construct, in the individuals tested when cell lines were stimulated with peptide pools. The interesting observation was made that when compared to the control wells of PBMC stimulated with uninfected DCs, the PBMC stimulated with adenovirus infected DCs reproducibly proliferated less (see figures 5.17 and 5.18 and data not shown of observations made while optimizing the growth of T cell lines to adenovirus infected DCs), although the DCs containing MV proteins stimulated more proliferation than the DCs containing β-galactosidase. This was interesting and it was decided to investigate this phenomenon further. This is the basis of chapter 6.

Chapter 6 Investigating the effect of adenovirus infection on DC function

6.1 Introduction

Following the observation made in chapter 5 that PBMC co-cultured with DCs which had been infected with adenovirus proliferated less than those co-cultured with uninfected DCs, it was decided that this phenomenon should be investigated further. Despite the wide use of adenoviral vectors in gene therapy and cancer immunotherapy protocols, as outlined in section 5.1.3, little is known of the functional consequences of adenoviral infection of human DCs.

6.1.1 The effect of adenoviral infection on cells

Previous studies have shown up-regulation of co stimulatory molecules such as CD80, CD86 and CD40 in response to adenoviral infection (Miller et al., 2002;Rea et al., 1999). These studies have also shown an increased capacity of infected DCs to produce IL-12 post CD40 ligation and therefore enhanced allo-stimulatory activity in a mixed lymphocyte reaction (Rea et al., 1999). Confirmation of these findings and further investigation of these phenomena in the mouse, was carried out by Morelli et al who showed that activation of DCs by adenoviral infection was via an NFκB dependent pathway (Morelli et al., 2000). In contrast other groups have reported no such up-regulation of co-stimulatory molecules on DCs infection with adenovirus (Tillman et al., 1999) and one group has reported a down regulation of MHC Class I on the infected DC surface post TNFα stimulation (Wold et al., 1999). These effects on DCs were therefore investigated in this adenovirus infection system. As no down regulation of co-stimulation or decrease in

costimulatory cyokine prouction was reported by these groups, it was anticipated that there would not be a problem as far as growing out T cell lines. In fact, if adenovirus infection did increase co-stimulation on the surface of the DCs in this system, it was predicted that adenovirus would improve the proliferation of T cells. However as described in chapter 5, T cell proliferation was reduced: this was therefore investigated, to characterize the effect of these adenoviral constructs on the phenotype and function of human DCs.

6.1.2 Adenovirus immune modulation

Previous work has shown that wild type adenovirus code for many genes that affect both acquired and innate immune responses (Horwitz, 2001). Most of these are within the E3 region of the viral genome which is deleted in the adenoviral constructs used for gene therapy (Horwitz, 2004), while others have been shown to be due to genes found in the E1 region (Wold et al., 1999). Both E1 and E3 regions are deleted in the constructs used in this study (Fooks et al., 1995). However recent findings have shown that despite the deletions in the E1 and E3 regions of the genome, adenoviral vectors can have a suppressive effect on the immune response in vitro when used to infect DCs (Tuettenberg et al., 2004).

Figure 6.1a) shows a schematic diagram of the adenoviral genome showing the different groups of genes. The E1 region is required for transcription of early genes which is deleted in the vectors used in this study. The deletions render the virus replication deficient, and it then requires the 293 packaging cell line for propagation since 293 cells are permanently transfected with the missing adenoviral genes required for replication. The gene of interest is placed under the CMV promoter in

place of deleted sections of genome (figure 6.1b)). There are however new generation vectors that are known as gutless vectors which have everything deleted except the inverted terminal repeats (ITR) and packaging sequences and these appear not to have the suppressive effect on DCs (Tuettenberg et al., 2004).

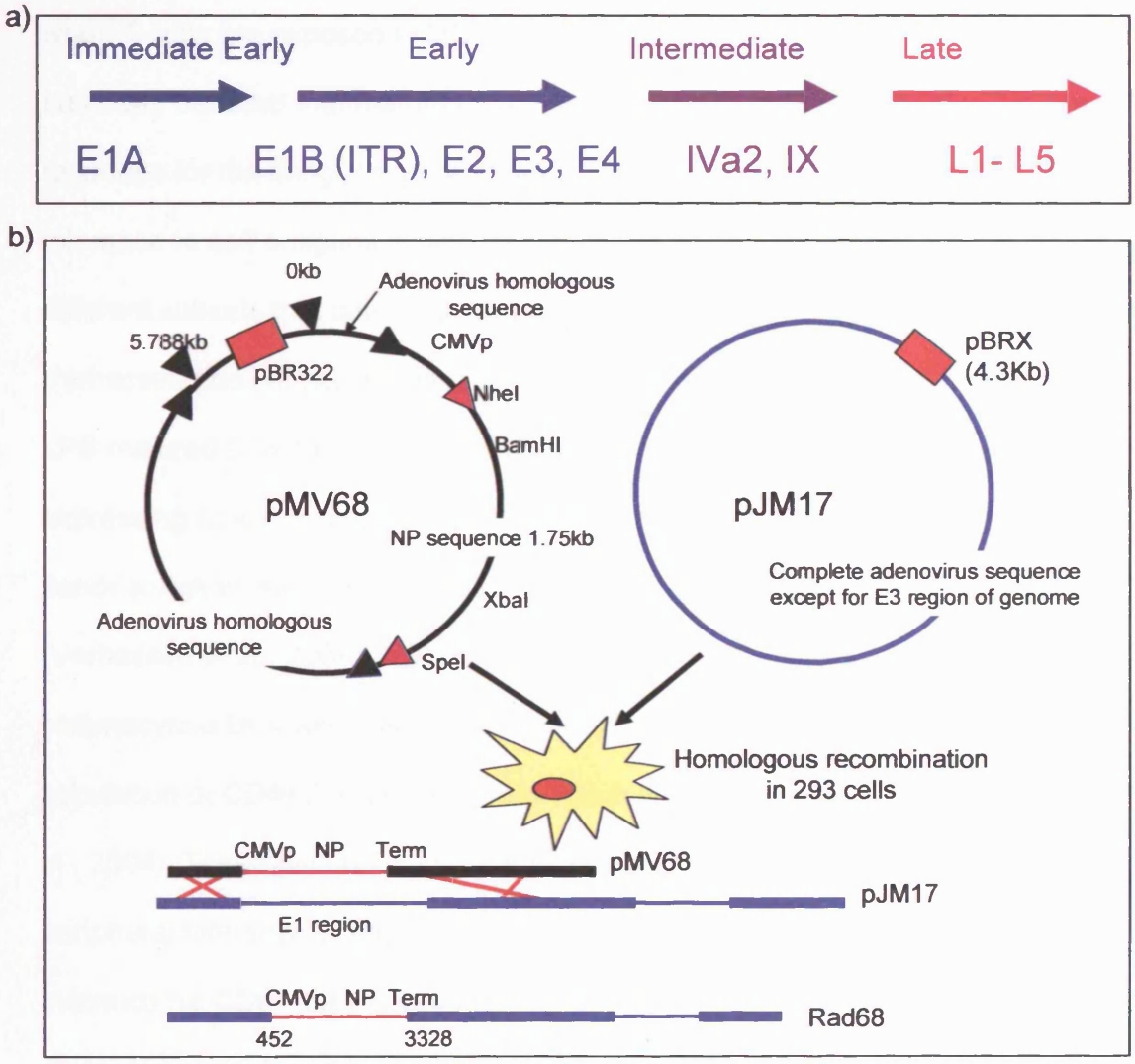


Figure 6.1: a) Schematic diagram of the adenovirus genome. The packaging sequence is a 400bp sequence upstream of the E1A region of the genome. b) Construction of the adenovirus recombinant genome containing the MV NP genetic sequence. The NP sequence under the CMV promoter was inserted into the ad transfer vector pXCX2 to generate pMV68. This provided the homologous Ad sequences to allow homologous recombination with the pJM17 plasmid to generate the recombinant viral genome of Rad68, the same principle applied for the generation of Rad35, 88 and 95.

6.1.3 DC maturation status and effect on resulting T cells

A large body of work has been generated recently relating to the role that the activation status and phenotype of DCs play in influencing the phenotype of T cells that have been co-cultured with those DCs. An interesting area of this research involves the induction of tolerance and generation of regulatory T cell subsets, when T cells are exposed to DCs in certain experimental situations. Traditionally it has been believed that mature DCs induce a specific and appropriate immune response for the danger signals received and in contrast, immature DCs induce tolerance to self antigens. However emerging data is identifying DCs of many different subsets that can induce a population of T regulatory cells (Treg). Verhasselt and colleagues have shown that in the absence of foreign antigen it is LPS matured DCs rather than immature, monocyte derived DCs that induce FoxP3 expressing CD4+ Treg cells in the responder population, which are specific for the donor antigens, since they do not suppress the proliferation of third party T cells (Verhasselt et al., 2004). A similar phenomenon has been observed in plasmacytoid DCs which when activated with CpG via TLR9 generate a similar population of CD4+CD25+ T reg cells which again express Fox P3 (Moseman et al., 2004). These data have implicated a role for mature DCs in the maintenance of peripheral tolerance during inflammatory and infection states. There is also evidence for CD8+ Treg generation in the gut by plasmacytoid DCs from the mesenteric lymph nodes (Bilsborough et al., 2003). *In vivo* studies in mice have shown that targeting of antigen to immature 'natural' DCs results in suppression of responses to that antigen on re challenge indicating another mechanism by which DCs can induce Treg in responder cells (Mahnke et al., 2003).

Whether the suppression of responses seen in systems where DCs have been infected with adenovirus is due to the generation of *de novo* Treg remains unclear. However the potential for mature DCs to induce the production of Treg is clear. Whether this is a viral escape mechanism that subverts the natural function of DCs for immune evasion or a natural consequence of infection under certain conditions is a question that requires investigation.

6.2 Specific materials and methods

6.2.1 Luminex

Supernatants from either mock or Rad35 infected DCs were harvested 24 hours post infection and frozen immediately at -80°C until use. Supernatants from proliferation assays with either mock or adenovirus infected DCs were harvested after 5 days and stored in the same manner. Cytokine measurements in these supernatants were made using the Luminex multiple cytokine detection kit (Upstate Biotechnology) according to the manufacturer's instructions. This assay is a bead based array based upon an ELISA principle, which can measure multiple ligands in one sample simultaneously (Khan et al., 2004)

6.2.2 Proliferation assays with DC

DC were generated as described in section 2.6.1.1. DC maturation was carried out using 100ngml^{-1} of PGE_2, IL-6, TNFα (all from Sigma UK) and IL-1β (Peprotech UK) as this was thought to induce maturation of DC in a less organism polarised manner to that achieved with LPS (Leen et al., 2004a). Proliferation assays were cultured in RPMI 1640 supplemented with antibiotics (as described in section 2.3) and 10% FCS (Invitrogen). DCs were either mock or adenoviral -infected and then

matured before use in proliferation assays. Responder cells were either allogeneic non adherent PBMC, or autologous non adherent PBMC. In autologous proliferation assays, cells were stimulated with 5μgml^{-1} PHA. Responder cells were labelled (prior to addition of DCs) for 10mins at 37°C in serum free medium containing 1μM CFSE (Sigma) as described in section 2.5.5. Cells were cultured at a ratio of 10:1 PBMC: DC for 5 days at a final concentration of 10^6 cells per ml before harvesting (see section 6.2.6).

6.2.3 Transwell experiments

Transwell experiments were performed in 24 well trans-well plates pore size 0.4μm (Corning) with responder cells and stimulus (either PHA for autologous cells or mock infected DCs for allogeneic responses) in the lower well at a concentration of 10^6 cells per ml and either infected or mock infected DCs in the upper insert at a concentration of 10^5 cells per ml.

6.2.4 Supernatant transfer

In supernatant transfer experiments, DCs were cultured for 24hours post infection with adenoviral constructs or mock virus preparation. Supernatants were harvested and added to autologous responder cells in place of culture medium at various dilutions as indicated.

6.2.5 Blocking antibodies and supplements

For blocking experiments either anti human TGFβ (clone 1D11 R&D Systems) or anti human IL-10 (clone 23738 R&D Systems) antibodies were added to the culture at a final concentration of 10μgml^{-1}. Recombinant human IL-2 was added to the

assay at dilutions from 10pg to 100ngml^{-1}. Recombinant human IL-10 was added to the cultures at a concentration of 20ngml^{-1}. The indoleamine 2, 3-dioxygenase (IDO) competitive inhibitor 1-methyl tryptophan was dissolved in DMSO and used at a final concentration of 250μM.

6.2.6 Cell analysis

After five days of culture cells were harvested and stained with anti-CD3-PE antibody (Becton Dickenson), washed three times, and fixed in 1% formaldehyde in PBS (Sigma) as described in section 2.5.1. Cells were run according to standard operating protocols on a Becton Dickenson FACScan (Becton Dickenson UK) with CellQuest software. Live cells were gated on CD3 to analyse proliferation of T cells only and 150,000 events were collected per condition. Proliferation was measured as the percentage of cells that had undergone more that one division as compared to the negative control of un-stimulated CFSE labelled cells.

6.2.7 Analysis of cytokine production by DCs

DCs were mock or adenovirus infected at an MOI of 50 for 24 hours before LPS was added for 2 hours prior to adding Brefeldin A for a further 14 hours as previously described in section 2.5.3. These cells were then harvested and stained as described in section 2.5.3 before being analysed against the cell surface marker DC-SIGN , a protein expressed on the surface of myeloid DC (Engering et al., 2002) for the cytokines TNFα, IL-6, IL-10 and IL-12.

6.3 Results

6.3.1 Maturation of DCs in response to adenovirus construct

There are conflicting reports as to the effect adenovirus infection has on DCs, as outlined in section 6.1. To investigate the effect of the adenovirus used in this study, DCs from 5 different healthy donors were cultured in medium alone, stimulated with LPS or infected Rad35. After incubation for 16 hours at 37°C/5% CO_2, phenotypic maturation was assessed using flow cytometric analysis of surface marker expression.

6.3.1.1 Surface expression on DC after infection with adenovirus constructs

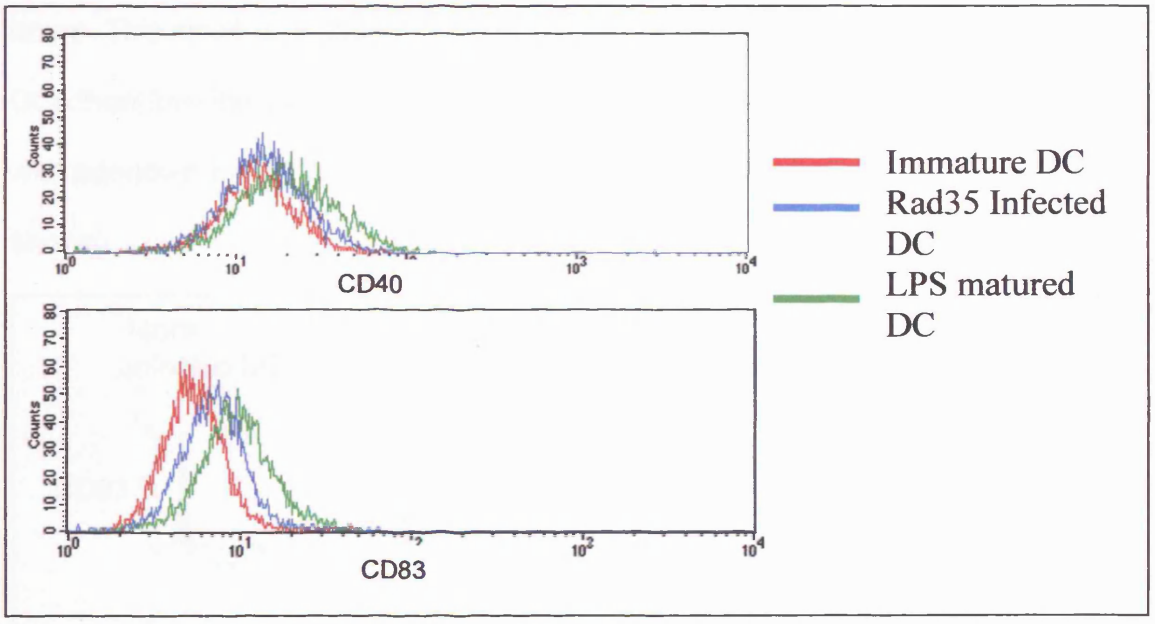


Figure 6.2 Flow cytometric analysis of DCs infected by Rad35 adenoviral construct. Histograms to give examples of partial maturation induced by Rad35 construct infection in one representative donor, AW7 as shown by partial up regulation of CD40 and CD83. Each marker is shown for immature DCs (red) DCs matured in LPS (green) and those infected with Rad35 (blue).

There was no single cell surface protein that changed in a consistent manner in all of the 6 donors. However there was a trend towards maturation with expression

levels increasing moderately but less than the change in LPS matured DCs. This was the case for markers such as MHC Class II DR, CD83, CD86 and CD40. Figure 6.2 shows this effect in two different markers (CD83 and CD40) in one representative donor (AW7). In all experiments the maturational changes of DCs were very similar for both Rad35 (βgal) and Rad68 (NP) constructs.

In order to test whether DC maturational changes in phenotype seen on adenoviral infection could be due to traces of LPS in the viral preparation, an experiment was performed where the equivalent level of endotoxin as measured in the viral prep ($25pgml^{-1}$) using the LAL assay (section 7.2.5) was incubated with DCs for 16 hours. This small concentration of LPS did not have an effect on the phenotype of DCs therefore the partial up-regulation of markers on the surface of DCs infected with adenovirus constructs was not due to contaminating levels of LPS (data not shown).

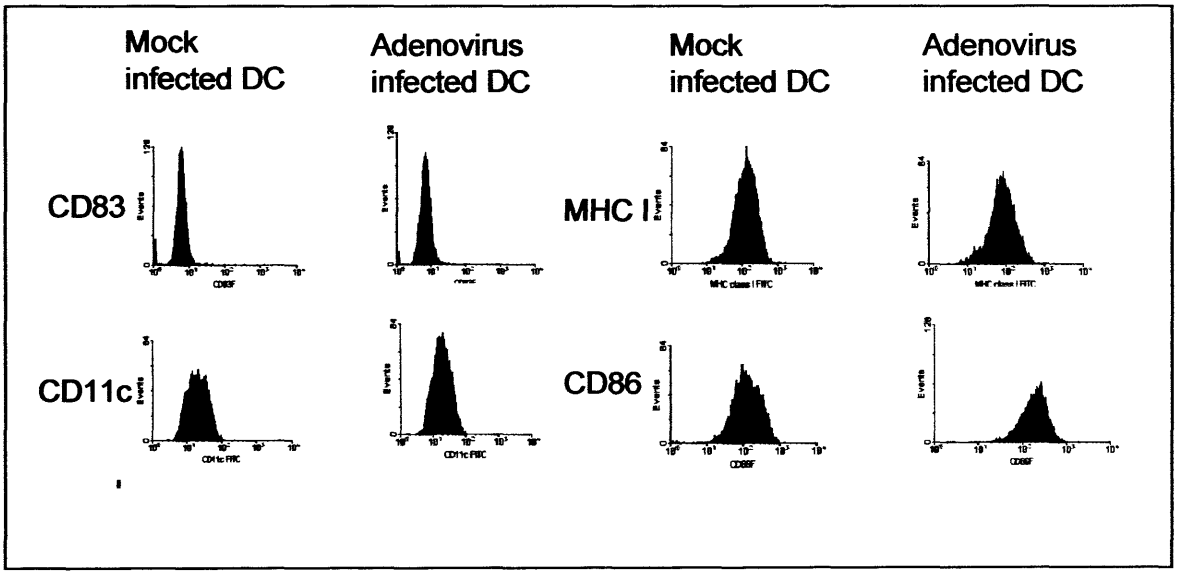


Figure 6.3 Histograms to compare the effect of adenoviral infection (Rad35) with mock infection on the phenotype of DCs from representative donor AW81. Histograms for markers CD83, CD11c, MHC I and CD86 are shown for this donor.

In order to establish if there were other contaminating agents within the virus preparation that may have an effect on the DC phenotype a mock preparation of virus was made. Figure 6.3 shows the results of this comparing DC surface expression of CD83, CD11c, MHC class I and CD86 on mock and adenovirus infected DCs in donor AW81. This donor has no change in CD83 and CD11c expression in response to adenovirus infection, however CD86 is up-regulated and in line with the findings of Wold et al MHC I is marginally down regulated (Wold et al., 1999).

6.3.1.2 Summary of different individuals

Table 6.1 shows a summary of the effect of adenovirus construct infection on maturation status defined by the expression of different markers in 5 different donors. The arrow represents the direction of the change in mean fluorescence intensity (MFI) of cells expressing the marker in the live DC gate.

Donor	CD80	CD86	CD83	DC SIGN	DR	CD40	CD14
AW84	↑	↑	↔	↓	↑	↔	↔
AW7	↑	↑	↑↑	↔	↑	↑	↔
AW5	↑	↑	↔	↓	↔	↔	↓
AW27	↔	↑	↑	↔	↑	↑	↔
AW80	↑	↑	↔	↓	↔	↑	↔

Table 6.1 Summary of phenotypic changes in DCs post adenoviral infection in five donors as assessed by cell surface expression levels of markers assayed by flow cytometry, 24 hours post infection. ↑ represents increased expression in infected cells, ↔ represents no change in expression and ↓ represents decreased expression of marker in adenoviral infected cells.

6.3.2 Cytokine production by DCs after infection with adenoviral constructs.

Both cytokine production in response to adenoviral infection and cytokine production in response to LPS stimulation post adenoviral infection were compared in 5 donors. After stimulation with LPS alone, adenoviral construct infection or both, DCs were analysed for intracellular cytokine production by flow cytometry as described in section 6.2.7. The results of this are shown in figure 6.4.

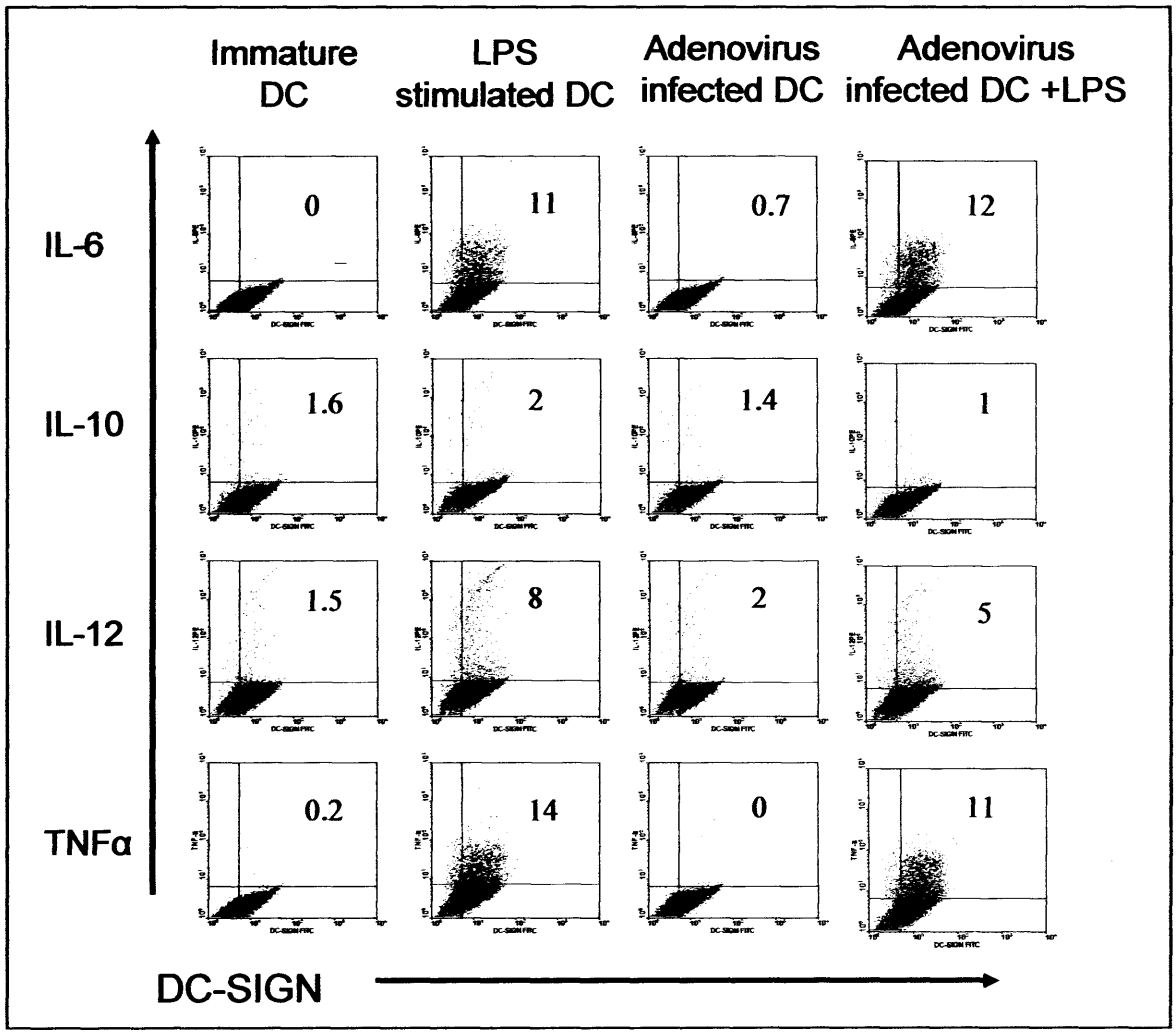


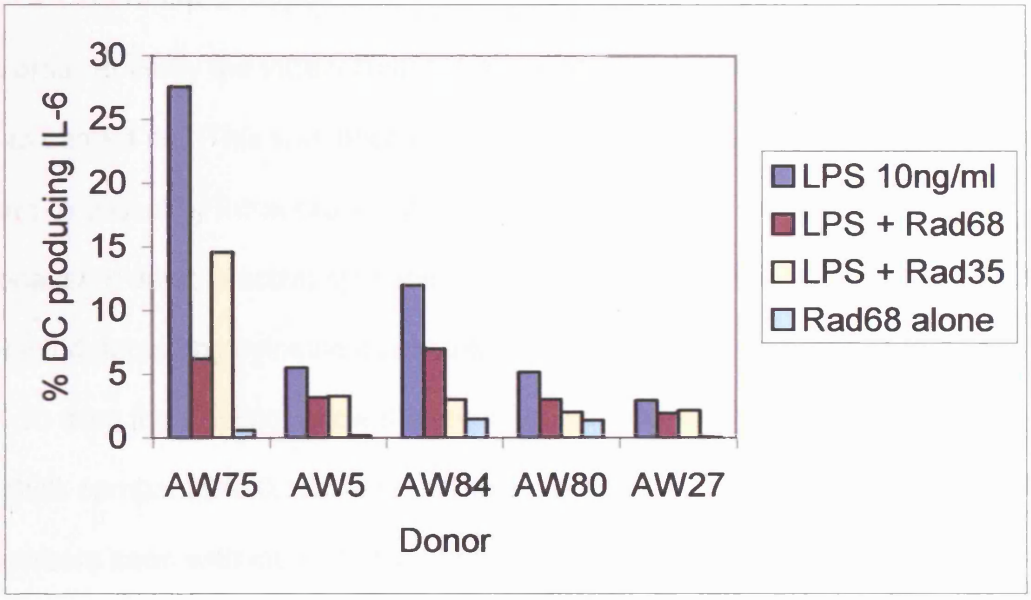
Figure 6.4: Cytokine production by DCs in response to adenoviral infection. Flow cytometric analysis of cytokine production by (from left) immature DCs, LPS (100ngml^{-1}) matured DCs, adenoviral infected (Rad35) DCs and both infected then matured DC (far right column) compared to immature cells and cells matured with LPS. The numbers in the upper right quadrants indicate the % of the live DC gate producing the cytokine indicated at the left of the figure.

In all donors, there was no significant increase in IL-12, IL-10, TNFα or IL-6 production in response to viral infection as shown in figure 6.4. In the donor represented in figure 6.4 the base line production is relatively high at 1.5% but this did not change on adenoviral infection with either Rad35 (figure 6.4) or Rad68 (data not shown). In each plot events are shown after gating on live dendritic cells. Cells have been stained with an antibody to DC SIGN and the antibody to the relevant cytokine.

6.3.2.1 Effect of adenoviral infection on cytokine response to LPS

Although there was no effect on cytokine production by DCs after adenoviral infection when 100ngml^{-1} LPS was used, it was thought this may be too strong to allow demonstration of any small effects due to virus. Therefore an alternative concentration of LPS, 10ngml^{-1} was used and as shown in figure 6.5.
From these data on five donors there is a trend towards both IL-6 and TNFα production in response to LPS being reduced post adenoviral infection (see for example AW75 and AW84). Repeated experiments need to be carried out to test the statistical significance of this, as the study is too small to reach a significant p value with these data.

a)



b)

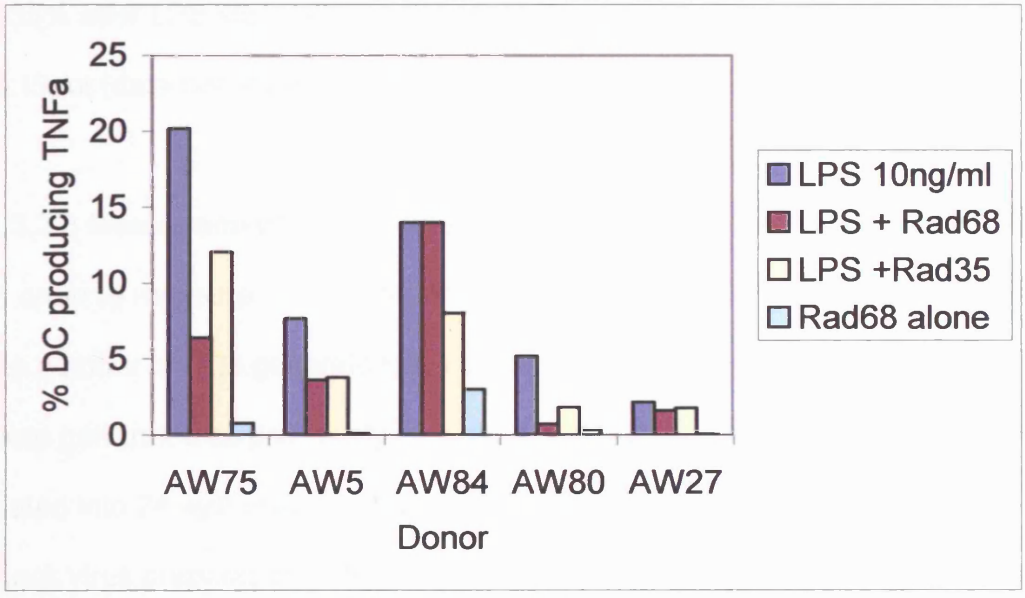


Figure 6.5 Graphs showing a summary of data from cytometric analysis of cytokine production by adenovirus infected DCs showing the difference in TNFα (a) and IL-6 (b) production on stimulation with LPS alone (10ng/ml), virus alone and response to LPS(10ng/ml) after adenoviral infection

6.3.2.2 IL-10 DC ELISpot

In order to verify the intracellular cytokine staining data for IL-10, an IL-10 ELISpot was carried out. This was because levels of IL-10 production were very low and hard to detect by intracellular cytokine staining. An ELISpot is a useful tool because it detects active cytokine secretion as opposed to intracellular staining which detects any cytokine inside the cell that may be being stored. The levels of IL-10 from the ELISpot show that even un-stimulated DCs are producing IL-10, 0.05% compared to 0.15% after stimulation with LPS. This is comparative to the numbers seen with intra cellular staining and using flow cytometric analysis. Intracellular staining shows 0.31% of DCs producing IL-10 before stimulation and 0.55% after LPS stimulation in the same donor (AW81) as measured using the ELISpot (data not shown).

6.3.2.3 Measurement of cytokines by multiple cytokine assay (Luminex)

In order to measure a much larger range of cytokines without having to increase the number of DCs generated, the Luminex multiplex bead assay was used. DCs were generated as previously described and on day 6 were harvested and re-plated into 24 well plates in 2ml medium. DCs were then infected with Rad35 or mock virus preparation. After 24 hours of incubation supernatants were harvested and stored at -80°C until use.

Table 6.2 shows the mean levels (+/- one standard deviation) for seven of the cytokines tested in 5 individuals from both adenovirus (Rad35) and mock infected DCs. IFNα, IL-5 , IL-12 and IL-13 were all below the level of detection this assay.

	Mean mock DC pg/ml +/- SD	Mean adeno DC pg/ml +/- SD
IFNγ	3.83 +/- 3.43	4.79 +/- 2.62
TNFα	1.37 +/- 0.62	2.45 +/- 0.62
IL-1β	0.39 +/- 0.29	0.79 +/- 0.71
IL-2	1.09 +/- 0.84	6.86 +/- 11.52
IL-4	426.19 +/- 350.35	512.18 +/- 515.46
IL-6	86.91 +/- 101.64	76.44 +/- 110.43
IL-10	0.15 +/- 0.11	0.43 +/- 0.20

Table 6.2 Measurement of cytokine production by DC as measured in supernatants after either mock or adenovirus (Rad35) infection, measured by the multiple cytokine assay (Luminex). Mean of 5 individuals +/- 1SD

6.3.3 Functional consequences of adenoviral infection of DC

As there did not appear to be a significant phenotypic difference between uninfected and adenoviral infected DCs, to address whether there were functional consequences to infecting DCs with adenovirus, the ability of adenovirus infected DCs to stimulate an allogeneic response was assessed. DCs prepared from one donor were infected with Rad35 or mock infected on day 6. On day 7 the DCs were matured using a cocktail of cytokines containing TNFα, IL-1β, IL-6 and PGE_2. It had been previously shown that the phenotypic maturation of the DCs achieved with this cocktail of cytokines was equivalent to that with LPS (data not shown); however it was felt that maturation using cytokines would lead to an activation of the DCs less skewed towards one type of organism. On day 8 the DCs were harvested and added in a ratio of 1:10 with PBMC from a different donor, which had been labelled with CFSE. Cells were then incubated for 5 days and then harvested and stained with anti CD3, for proliferation of the T cells to be assessed. The effect of adenovirus infection on allogeneic responses is shown in figure 6.6. Figure 6.6 a) shows a representative experiment AW81 responder cells with AW89 DCs and b) shows pooled data from 12 individual experiments with different donor

pairs. A paired t test was carried out on these data showing statistical significance of $p<0.0005$ between proliferation induced by mock compared to adenoviral infected DCs.

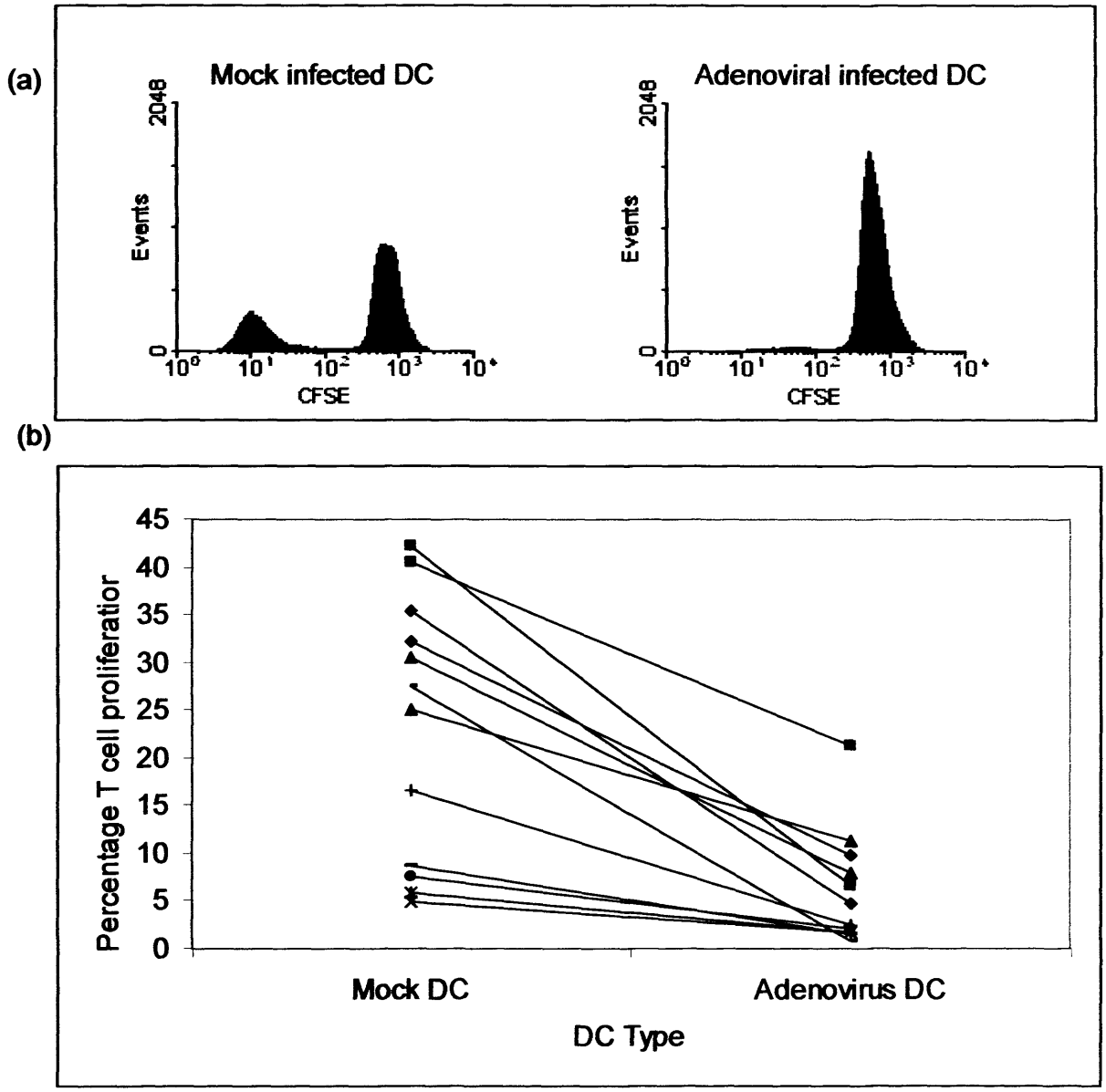


Figure 6.6: The effect of adenoviral infection on an allogeneic response. Flow cytometric analysis of CFSE labelled cells in an allogeneic proliferation assay. a) Representative CFSE plot (AW81 responder cells with AW89 DC 1 of 12 independent experiments b) The differences between proliferation to mock and adenoviral infected DC are highly significant ($p<0.0005$).

6.3.4 Active suppression of response to PHA

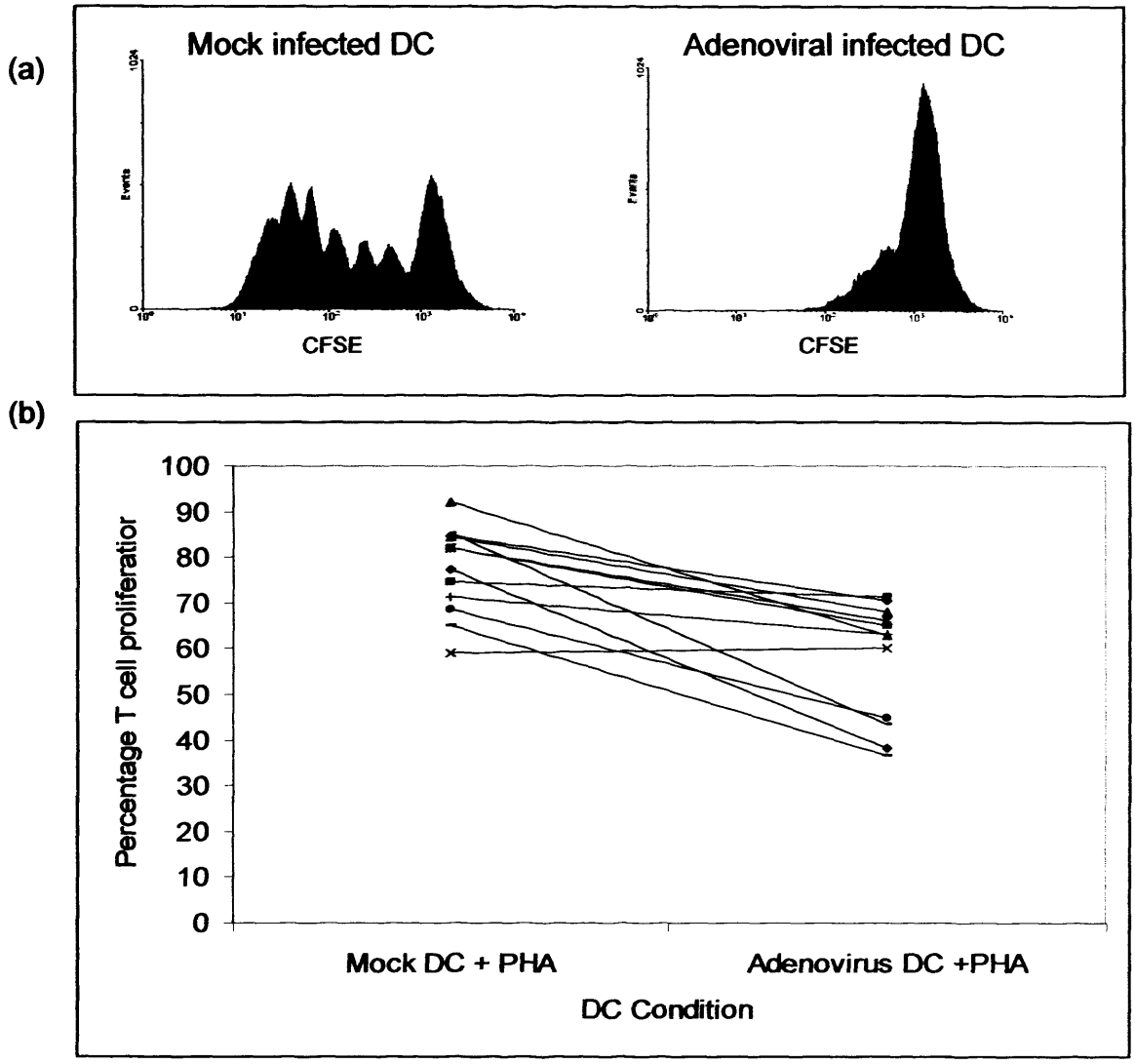


Figure 6.7: The effect of adenoviral infection on the T cell response to the mitogen PHA. Flow cytometric analysis of CFSE labelled cells in a proliferation assay to PHA. a) Representative CFSE plot (AW81) of 12 independent experiments (b) The differences between proliferation to mock and adenoviral infected DCs are highly significant ($p<0.0005$).

As it had previously been shown that there was no increased death of DCs (see section 5.3.3.3), no significant down regulation of any co-stimulatory molecules (in fact the opposite) and no significant inhibition of ability to make cytokine on stimulation post adenoviral infection of DCs, it was decided to test the possibility that the observed phenomenon was not in fact due to a DC defect but was due to

an active suppression of proliferative responses by the adenoviral infected DCs. In order to do this, DCs were generated as for the allogeneic experiment. However autologous responder cells were added instead of allogeneic cells. PHA was also added to the culture, this mitogenic lectin stimulates the T cells to proliferate but the effect would not be dependent on the additional DCs to allow the proliferation. Figure 6.7 shows that the adenovirus infected DCs actively suppress non specific proliferative T cell responses to PHA. Figure 6.7a) shows one representative donor (AW81). While in figure 6.7b), 12 independent experiments are summarised. Using a paired t test the data was analysed again reaching a significance value of $p<0.0005$, comparing proliferation to PHA in the presence of mock or adenoviral infected autologous DCs.

6.3.5 Adenoviral infection of monocytes can also suppress T cell responses. To investigate whether this phenomenon was restricted to DCs, or whether other cell types could mediate suppression, PBMC were allowed to adhere to plastic for two hours, non adherent cells were removed, then cells were infected with the adenovirus constructs. After 24 hours cells were harvested and co-cultured with T cells at a ratio of 10:1, as previously described for co-culture with DCs (section 6.2.2). This fraction of cells (predominantly monocytes) was able to suppress the proliferation of autologous T cells to PHA as shown in figure 6.8. In addition by infecting one T cell population with adenovirus, and culturing this population with T allogeneic T cells in a traditional MLR, T cell proliferation was also suppressed in the responder T cell population (data not shown). Both these phenomena show that the ability to suppress proliferation of T cells upon adenoviral infection with these constructs is not confined to DCs.

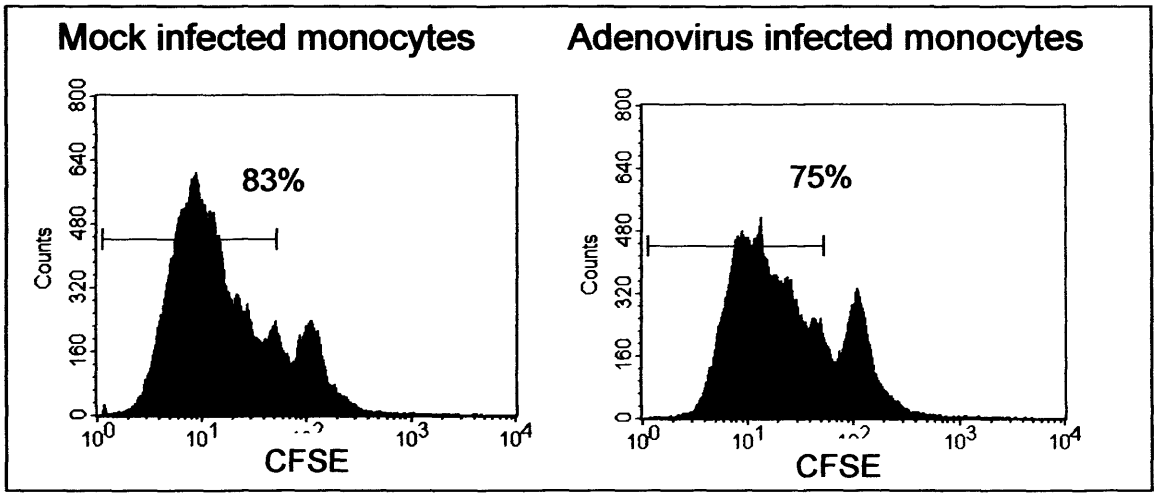


Figure 6.8: Flow cytometric analysis of CFSE labelled T cells showing that adenovirus infected monocytes suppress T cell proliferation to PHA in one representative donor (AW81) out of 2 donors.

6.3.6 Suppression by adenovirus infected DCs is not reversed by addition of IL-2

Although no consistent change in IL-2 production was seen in the supernatants from infected DCs, (table 6.2), in certain individuals IL-2 was reduced in supernatants collected from adenovirally infected DCs compared to mock infected DCs (table 6.2). Using cells from such a donor (AW5), and given that some 'regulatory' functions seen in *in vitro* assays may be overcome by addition of exogenous IL-2 (Takahashi et al., 1998), recombinant IL-2 was added to the culture of T cells and DC in the PHA assay, at a range of concentrations from 10pg to 100ng/ml, to investigate whether exogenous IL-2 would prevent the inhibitory effect of adenovirus- infected DCs. However the addition of h rIL-2 did not overcome the suppression (figure 6.9), even up to a concentration of 100ng/ml of exogenous IL-2.

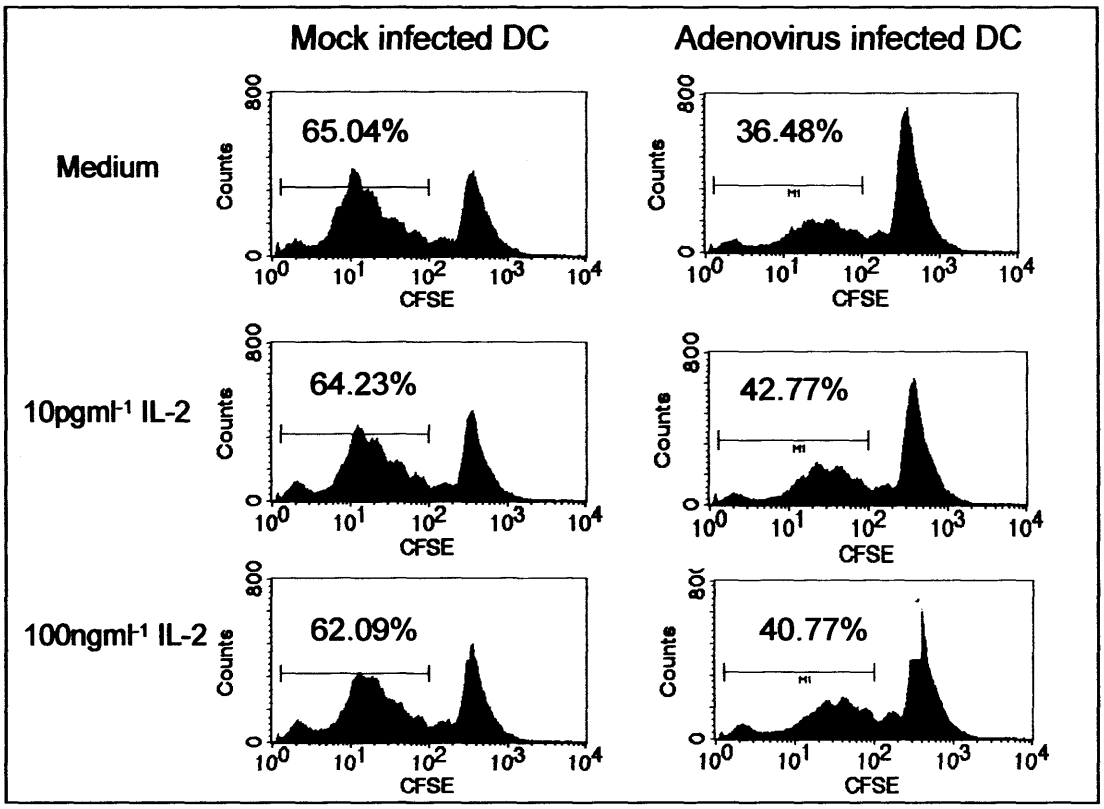


Figure 6.9: IL-2 has no effect at overcoming suppression. Flow cytometric analysis of proliferation of CFSE labelled cells incubated with either mock (left panel) or adenoviral (right panel) infected DCs with IL-2 titrated into the culture system between 10pg and 100ngml^{-1}, this has no effect on the suppression by adenovirus infected DCs (1 of 2 experiments shown from donor AW5).

It was then tested whether T cells would proliferate when infected with adenovirus themselves. The results of this for one representative donor (AW5) of three are shown in figure 6.10. This showed that small numbers of T cells do proliferate to adenovirus. However proliferation of infected T cells to PHA was considerably reduced when compared to mock infected T cells. These data in figure 6.10 show that the suppression of T cell proliferation that has been observed, may not be restricted to an infected population inhibiting proliferation of an uninfected population but that the suppression can also occur within a population of cells that

have been infected. It is not likely that the uninfected cells in the population are being affected as infection of PBMC was typically 95% (see section 5.3.3). Therefore, not only can infected cells inhibit proliferation in other cells but they themselves are prevented from proliferating.

In order to ensure that the protein β-galactosidase which is expressed in Rad35, the construct used for these experiments was not responsible for the observed suppression, the same experiment as in section 6.3.4 was carried out using a lentiviral vector containing β-galactosidase kindly provided by Barry Flutter. DCs infected with this construct had no effect on the proliferation of T cells to PHA leading to the belief it is an adenovirus specific phenomenon (data not shown).

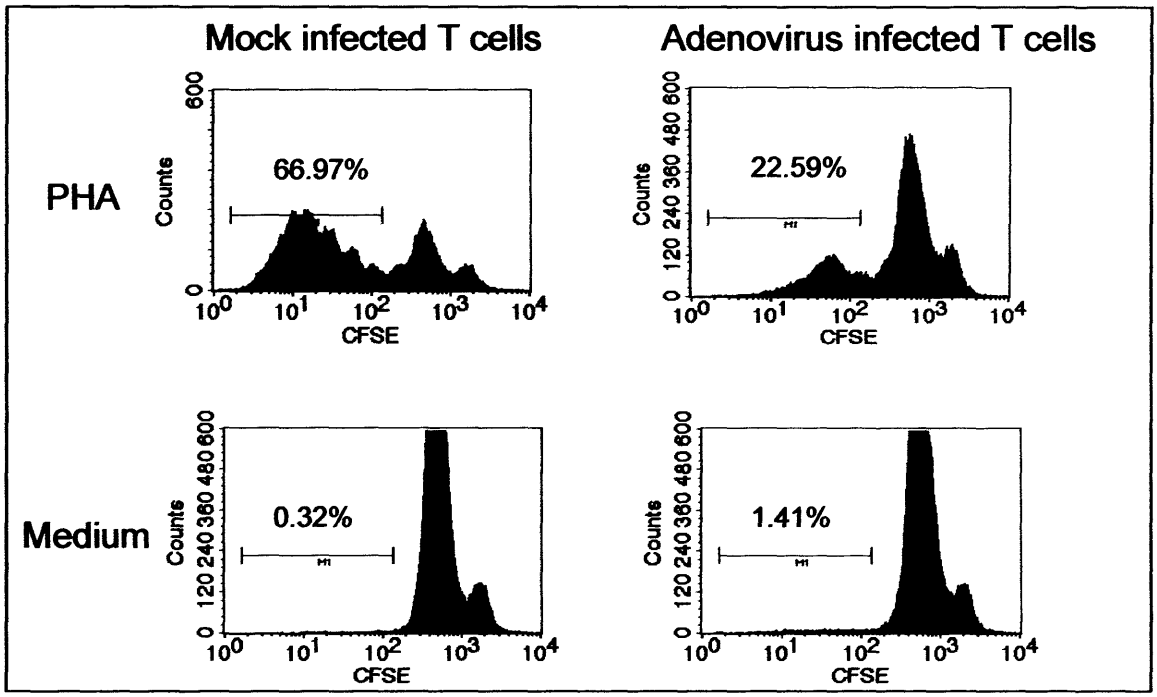
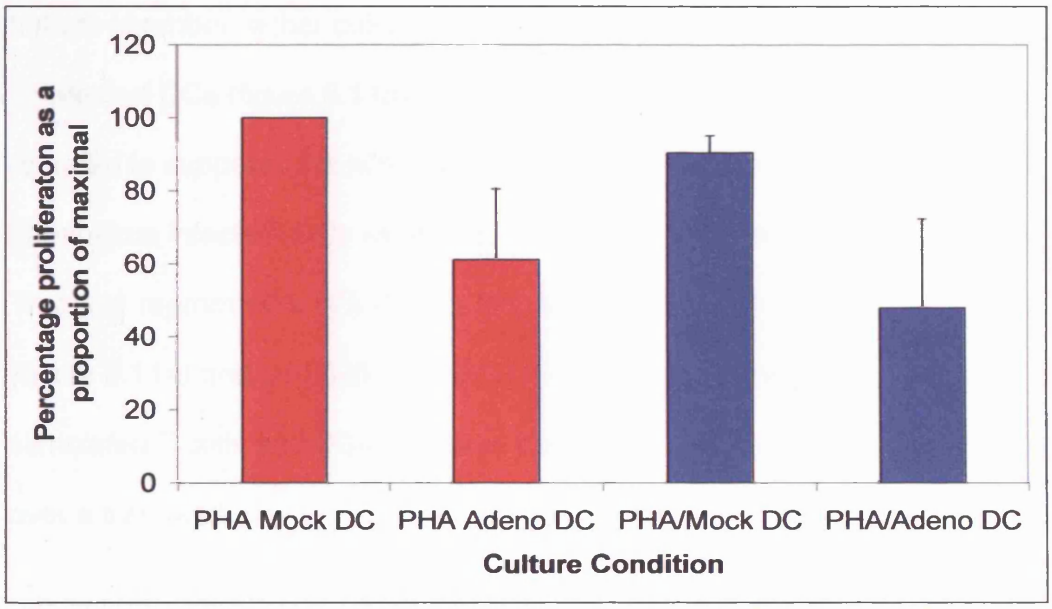


Figure 6.10: Proliferative response of T cells to adenovirus infection. Flow cytometric analysis of CFSE labeled cells that have been infected with adenovirus and incubated for 5 days with or without PHA. 1% of T cells proliferate in response to being infected with adenovirus (bottom panels) yet proliferation of infected cells to PHA is impaired (top panels) (1 of 3 experiments shown).

6.3.7 A soluble factor (or factors) is responsible for the suppression seen with adenoviral infected DCs.

a)



b)

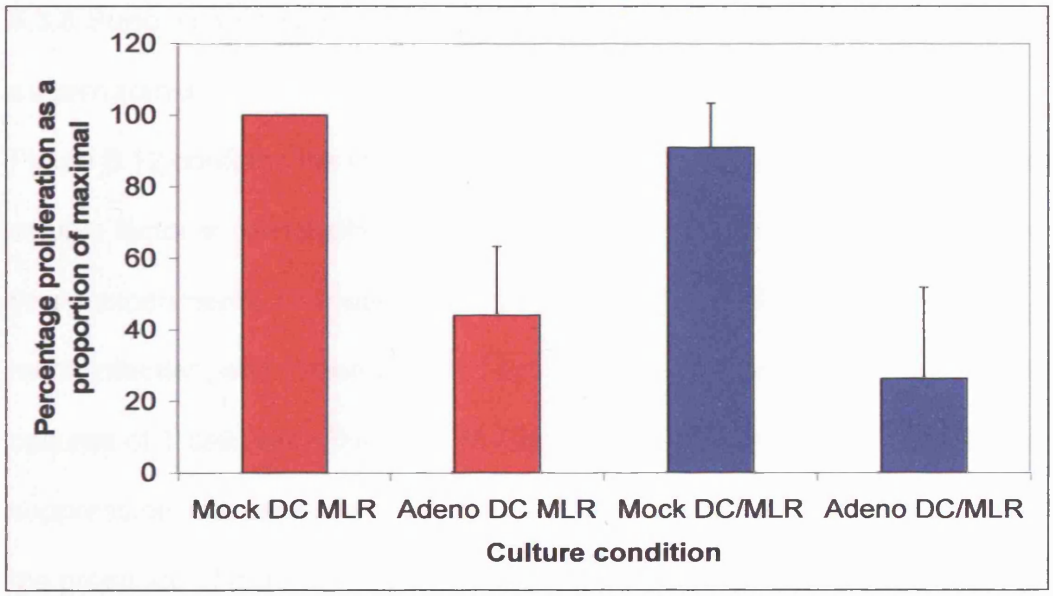


Figure 6.11: Summary of data from CFSE labeled cells in a proliferation assay after transwell culture. A soluble factor is responsible for the suppression of T cell proliferation. Percentage proliferation with DC/T cell contact (red bars) and without contact (blue bars) when T cells were stimulated a) with PHA and b) in an allogeneic response. Data represent mean values from 5 donors, with error bars showing one standard deviation.

To test whether this phenomenon was cell contact dependent or could be mediated by soluble factors, a transwell system was used, where the DCs (either infected or uninfected), were placed at the top of the transwell, with responder cells in the bottom chamber, either cultured with PHA (figure 6.11a) or with a population of uninfected DCs (figure 6.11b). This system was used to test whether contact was required to suppress the allogeneic response, as well as the response to PHA. The adenovirus infected- DCs were able to suppress both responses over a transwell. This was reproducible in 5 donors and reached a significance value of $p<0.01$ (figure 6.11a) and $p<0.025$ (figure 6.11b), when comparing co-cultures of stimulated T cells and DCs that have been mock or adenoviral infected separated over a transwell .

6.3.8 Suppressive activity can be transferred by adenovirus infected DC supernatants.

Figure 6.12 confirms the results seen in section 6.3.7 by again showing that a soluble factor is responsible for the suppression by adenovirus infected DCs. In these experiments, DC supernatants were harvested 24 hour post infection or mock infection, and frozen at -80°C until use. Supernatants were then added to cultures of T cells with PHA at 50% this was repeated in 3 donors (figure 6.12) and suppression was observed in all cases when comparing proliferation of T cells in the presence of mock infected DC supernatant to those proliferating in the presence of adenovirus infected DC supernatant. Supernatants were then titrated into T cell/PHA cultures showing that the suppressive effect was concentration dependent (figure 6.13).

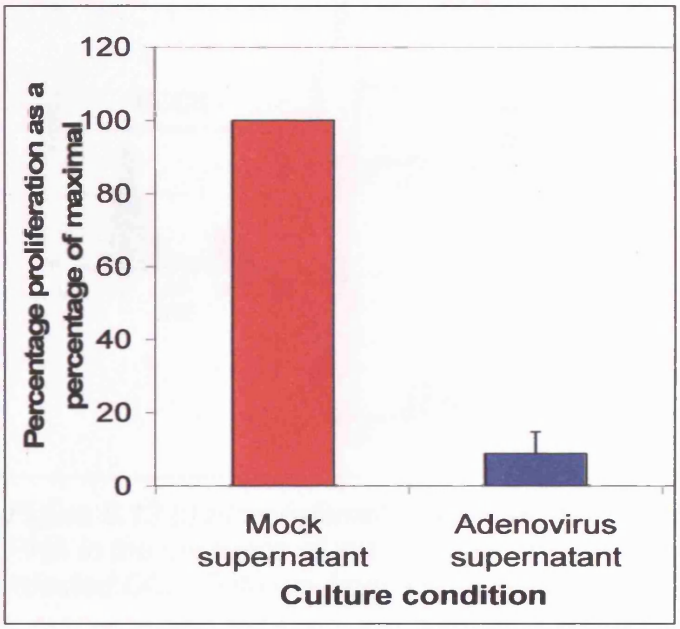


Figure 6.12: Effect of addition of supernatant from mock infected DCs (red bar) or adenoviral infected DCs (blue bar) on T cell proliferation to PHA. Assayed by CFSE labeled cell proliferation analysed by flow cytometry. Bars represent mean values from 3 donors and error bars are one standard deviation.

a)

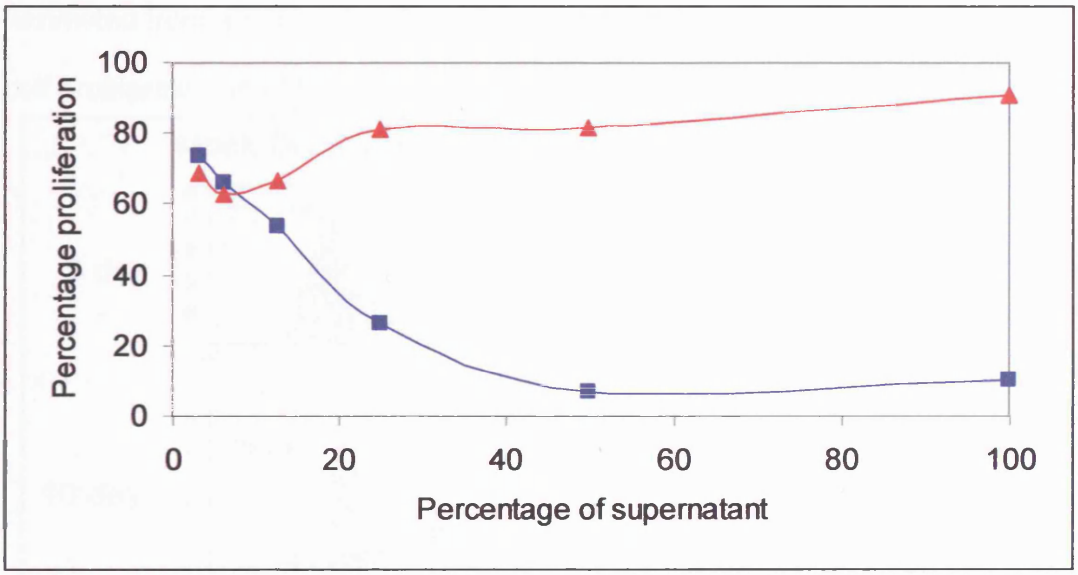


Figure 6.13 a) Effect of titrating supernatant from mock (red) or adenovirus (blue) infected DCs into the culture of T cells and PHA a) Graphical representation of the effect of mock and adenovirus infected DC supernatants on the proliferation of CFSE stained T cells to PHA in a five day culture as analysed by flow cytometry.

b)

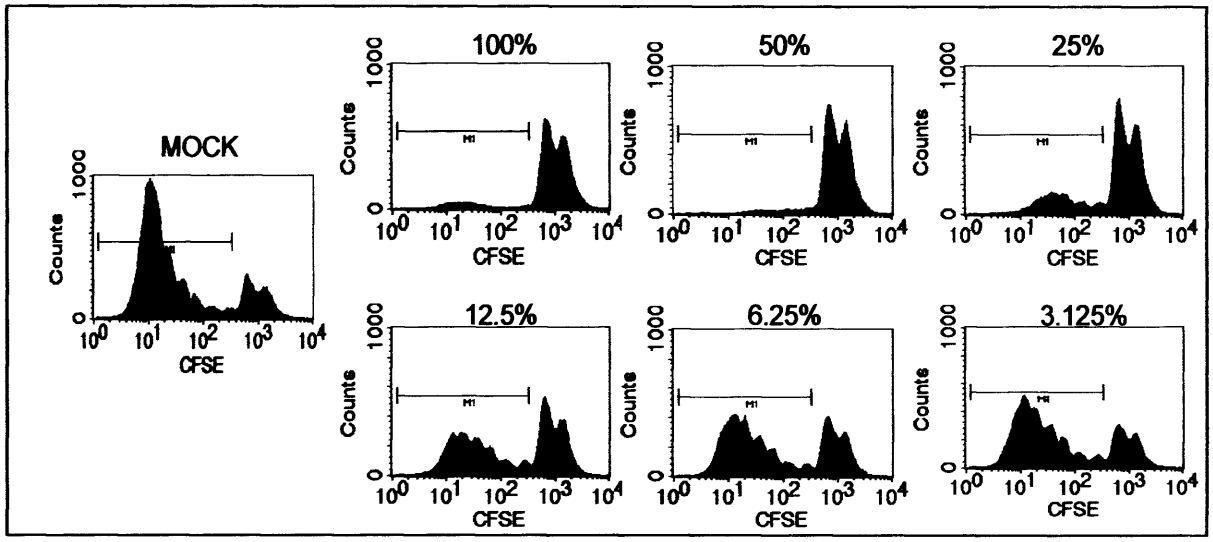


Figure 6.13 b) Flow cytometry plots showing CFSE labeled T cells proliferating to PHA in the presence of increasing dilutions of supernatant from adenovirus infected DCs. Data are from one representative (AW88) of two donors tested.

6.3.9 5 and 10 day supernatants also suppress T cell responses

Contrary to the suggestion of others that the factor involved in suppression was only active early in the infection process(Tuettenberg et al., 2004), supernatants harvested from adenovirus infected DCs, after both 5 and 10 days suppressed T cell proliferation to PHA as shown in figure 6.14.

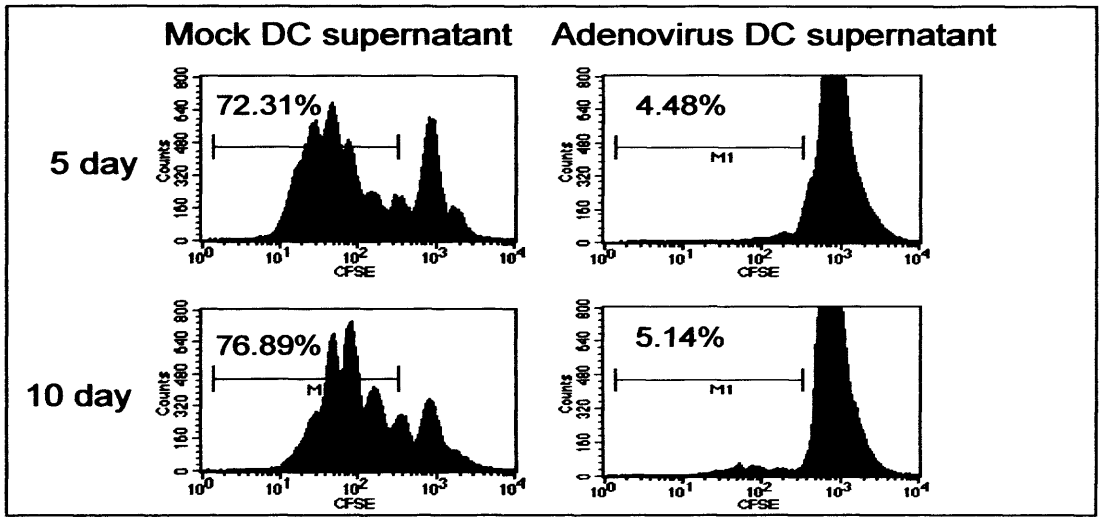


Figure 6.14: 5 and 10 day supernatants also suppress proliferation. Flow cytometry plots showing CFSE labeled T cells proliferating to PHA in the presence of 50% supernatant from adenovirus infected DCs after 5 and 10 days. Data are from one representative (AW89) of three donors.

6.3.10 Blocking inhibitory cytokines IL-10 and TGF-β

To address the question of whether an inhibitory cytokine was responsible for the suppression, blocking antibodies to IL-10 and TGF-β were used in the PHA proliferation assay, to look at the role that these cytokines may play in the suppression. IL-10 appeared to be involved in the suppression in one out of 4 donors tested, as blocking partially restored proliferation to PHA (figure 6.15). Blocking TGF-β had no effect in any of the donors (figure 6.15 and data not shown).

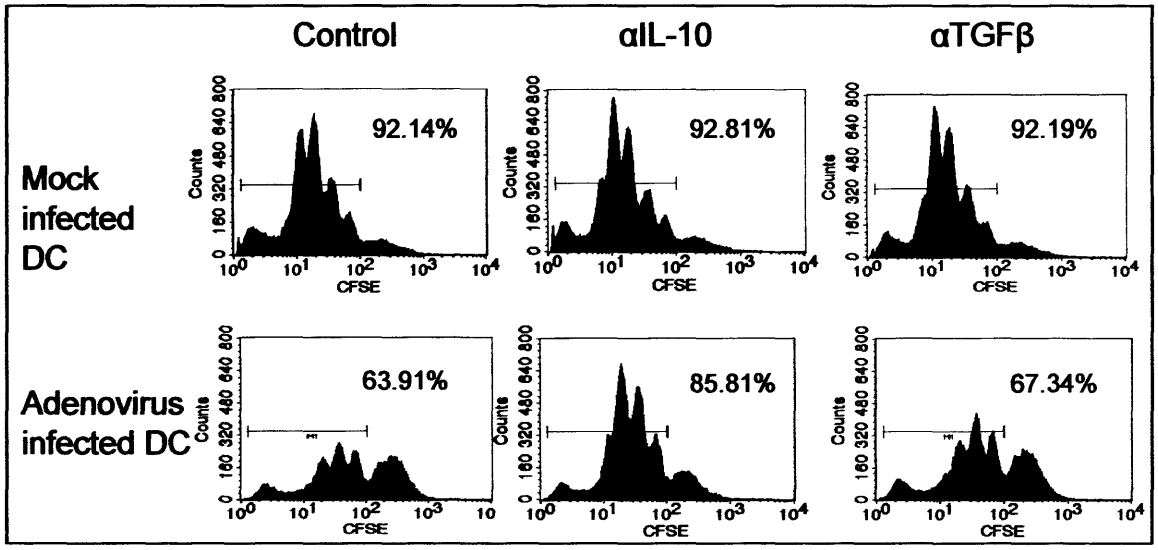


Figure 6.15 Blocking IL-10 reverses the suppression in one donor. Histogram plots of T cells stained with CFSE and stimulated with PHA co-cultured with mock and adenoviral infected DCs and blocking antibodies, showing restoration of proliferation in one donor by addition of an anti IL-10 blocking antibody to the T cell/PHA culture. One individual donor of four tested that had this effect.

As an increase in IL-10 production was not detected in DCs post adenoviral infection (table 6.2), cytokine production in the proliferation assay was measured in 2 individuals. Supernatants from PHA proliferation assays were harvested after 5 days and analysed using the multiplex bead assay (Luminex). An increase in IL-10

production in the cultures with adenovirus infected DCs was seen when compared to mock infected DC cultures (table 6.3). IL-1β also increased and levels of IL-5 and IL-13 were reduced (table 6.3). However further experiments of this type will need to be performed to increase the power of these observations, and determine their significance.

	PHA Mean pg/ml +/- SD	PHA + ad DC Mean pg/ml +/- SD	PHA + Mock DC Mean pg/ml +/- SD
IFNγ	479.61 +/- 550.62	11980.23 +/- 3087.67	12943.57 +/- 18304.94
TNFα	20.57 +/- 17.25	755.34 +/- 76.51	1019.62 +/- 533.81
IL-1β	1.19 +/- 0.24	312.27 +/- 15.32	51.69 +/- 15.1
IL-4	3.70 +/- 5.23	130.47 +/- 180.42	42.34 +/- 12.79
IL-5	47.53 +/- 65.07	228.53 +/- 87.72	790.22 +/- 240.9
IL-6	196.83 +/- 169.27	1577.27 +/- 2230.59	912.17 +/- 1290
IL-10	20.59 +/- 27.9	305.11 +/- 50.02	193.55 +/- 14.86
IL-12	0.00 +/- 0	41.20 +/- 8.05	55.18 +/- 67.25
IL-13	136.46 +/- 184.58	675.46 +/- 368.57	2032.11 +/- 249.13

Table 6.3: Luminex measurements of mean cytokine levels (+/- one standard deviation) from 2 donors in supernatants from T cells cultured with PHA, PHA and mock infected DCs and PHA and adenovirus infected DCs

6.3.11 Addition of IL-10 and inhibition of IDO

Recently the work of Tan and colleagues has implicated a role for indoleamine 2,3-dioxygenase (IDO) in the reduced response of T cells in MLR with adenovirus infected DCs. Therefore the role of IDO in this system was investigated, by adding the IDO competitive inhibitor 1-methyl tryptophan as described in section 6.2.5. However in the 3 individuals tested, one of which is shown in figure 6.16 IDO did not appear to play a role in this system. Figure 6.16 also shows that adding back IL-10 at a concentration of 20ngml^{-1} has no effect on the proliferation of cells and does not cause a suppression of proliferation when added alone, indicating there must be another mediator or pathway involved in mediating the suppression. These experiments are preliminary and must be extended to larger numbers of

samples in order to gain a true representation of what mechanism the T cells are suppressed by including investigating the phenotype and function of the T cells generated through co-culture with infected DCs.

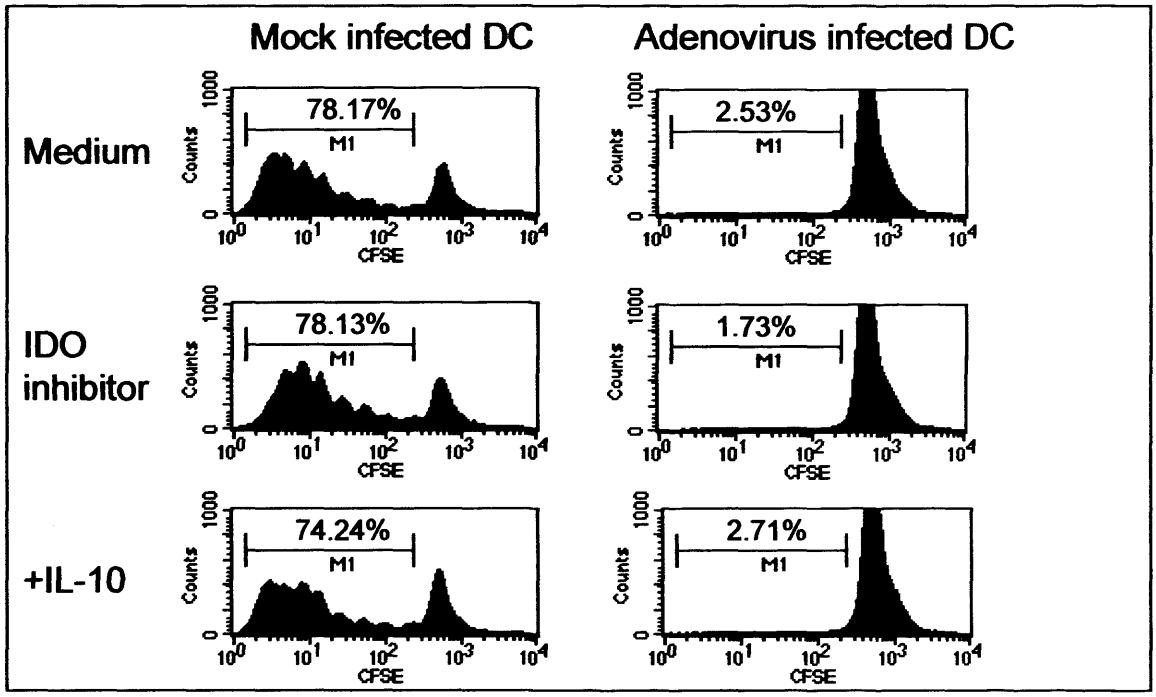


Figure 6.16: Effect of IDO inhibition on suppressive effect of adenoviral infected DCs. The IDO competitive inhibitor 1-methyl tryptophan or recombinant human IL-10 was added to T cells labelled with CFSE and cultured with PHA and either mock or adenovirus infected autologous DCs. Analysed by flow cytometry (1 (AW89) of 3 individuals tested).

6.4 Discussion

The data presented here show that adenoviral infection of DCs using the constructs containing either MV-NP (Rad68) or β-galactosidase (Rad35) induced a small degree of maturation in DCs as measured by up regulation of surface co stimulatory markers, such as CD83 and CD40. This study also found no significant change in cytokine production by adenoviral infected DCs either with MV NP protein construct or βgal protein construct, once stimulated with LPS. These findings are in agreement with studies that have also shown up regulation of co

stimulatory molecules such as CD80, CD86 and CD40 in response to adenoviral infection (Miller et al., 2002;Morelli et al., 2000;Rea et al., 1999).

This study contributes novel information to our current knowledge of how viruses, and specifically adenoviral constructs, may affect dendritic cell function. These results show that in human monocyte-derived immature DC (iDC), infection with Ad5 E1 and E3-deleted adenovirus leads to a reduced capacity of the DCs to stimulate T cells, whether these are stimulated in an allogeneic manner (figure 6.6) or by the mitogen PHA (figure 6.7). In the PHA-driven response, additional DCs are not required for responder cells to proliferate: therefore reduction of proliferation seen on the addition of adenovirus-infected DCs cannot be due to a DC defect, but instead suggest an active inhibition, mediated by the infected DCs. These results are compatible with previous reports of reduced allo- stimulatory capacity of adenoviral infected DCs in humans (Jonuleit et al., 2000;Tan et al., 2005;Tuettenberg et al., 2004). However both these studies, and the data presented here conflict with earlier evidence, which suggested an enhanced capacity to stimulate T cells by DCs infected with adenovirus, an effect attributed in part to increased expression of co stimulatory molecules CD80, 86 and 40 (Rea et al., 1999;Zhong et al., 1999). These results are the first to demonstrate formally that the suppressive effect of some adenoviral constructs is a dominant inhibition, since it is seen in both an allogeneic stimulation assay and also in the response to the mitogen PHA. In this system, the inhibitory effect was very powerful, as it was clearly demonstrable at ratios of T: DC as low as 50:1 (data not shown). Most previous studies of a parallel type have used cells at a T: DC ratio of 10:1. In addition to this the results show that the suppression can be mediated by cell types

other that DCs such as monocytes, although suppression using monocytes at an equivalent ratio was less powerful.

In line with previous reports of increased expression of CD80, 86 or other cell surface molecules after adenovirus infection of iDCs (Rea et al., 1999;Tan et al., 2005;Zhong et al., 1999), the data presented here shows small changes in the phenotype of DCs upon infection by adenovirus, including small increases in expression of CD80 CD86, CD83, CD40 and HLA-DR in some individuals, indicating a trend towards maturation upon adenoviral infection. In addition to investigating the phenotype of DCs after infection by adenoviral constructs these cells were analysed for cytokine production. There was no production of IL-12, IL-6, IL-10, TNFα, or IFNγ from infected DCs when cultured alone. In addition, infected DCs were able to mount a strong response to the TLR4- signal by addition of LPS, as analysed by intracellular cytokine staining (figure 6.4). Multiplex cytokine measurements on supernatants collected from a total of 5 individuals confirmed these results. Some earlier reports also suggested no change in cytokine production by DCs infected with adenovirus (Rea et al., 2004) but a more recent study has suggested up-regulation of many type-1 and pro-inflammatory cytokines in this system (Tan et al., 2005). Data from work in mice are also conflicting with some reports suggesting IL-12 production post adenoviral infection (Miller et al., 2002). However others show production of IL-6, IL-15, IFNγ and TNFα in response to adenovirus infection but without IL-12 and IL-10 production (Morelli et al., 2000;Philpott et al., 2004).

Small but critical differences in reagents or protocols may account for these discrepant results, such as the exact gene content of the adenoviral constructs, or the timing of infection and agents to induce DC maturation. Thus, for example, the fact that 'gutless' vectors confer less change on DC phenotype, and also that active adenoviral protein synthesis is required to see changes in DC expression following infection, implicating specific adenoviral products in these effects (Tuettenberg et al., 2004).

With respect to the mechanism of inhibition conferred by adenoviral infected DCs in this study, the results demonstrate that suppression of T cell proliferation with these constructs is largely mediated by soluble factors. Thus supernatant from the infected DCs transferred inhibition, and when DCs and T cells were separated by a transwell which prevented cell contact, inhibition was still seen in both assays (figure 6.11). In contrast, previous studies have suggested that the inhibition mediated by adenoviral infected DCs requires cell-cell contact (Jonuleit et al., 2000). The production of IL-10 by DCs after viral infection has been described for several other viruses, in particular measles, (Servet-Delprat et al., 2000b) influenza, (Oh and Eichelberger, 2000) and RSV (Bartz et al., 2002). Therefore it was considered that adenovirus infection with these constructs may also alter cytokine production by DCs and thereby have an inhibitory effect on T cell stimulation.

Although no clear candidate cytokine was identified in the supernatants from infected DCs, two possible soluble factors which may be mediating the inhibition seen in this study were investigated. This showed that at least in one individual,

blocking of IL-10 (but not TGFβ) partially reversed the inhibitory effect of adenovirus infected DCs, on the T cell response to PHA. However intracellular cytokine staining of DCs and cytokine assays of supernatants from adenovirus-infected DCs failed to detect the production of IL-10 by infected DCs. The finding that blocking IL-10 reversed the suppression in some donors, yet it could not be detected in adenovirus-infected DCs indicates that IL-10 may not be the predominant or sole DC- derived soluble factor responsible for the suppression. It does however implicate IL-10 as a possible effecter molecule in one of several possible pathways that may be induced by these constructs. This also raises the possibility that as the IL-10 is produced not by the DCs in this system, but by T cells within the responding population. One mechanism for this effect may be the induction of IL-10 production in a population of the T cells, perhaps of a Tr-1 like phenotype (Levings et al., 2005;Roncarolo et al., 2001;Wakkach et al., 2003).

Recent work by Tan and colleagues has implicated a role for indoleamine 2,3-dioxygenase (IDO) in the reduced response of T cells in MLR with adenovirus infected DCs. Therefore the role of IDO in this system was investigated. IDO is an enzyme that degrades the essential amino acid tryptophan. Tan et al found that adenovirus infected DC inhibited T cell proliferation in an MLR and that blocking the activity of IDO restored proliferation (Tan et al., 2005). One mechanism of IDO action is to inhibit T cell proliferation by depriving them of tryptophan. Cells have been shown to undergo cell cycle arrest when deprived of tryptophan (Lee et al., 2002;Munn et al., 1999) which would relate well to the work of Tuettenberg et al who show T cells arrest in G1 phase after being exposed to Adenovirus infected DC (Tuettenberg et al., 2004). Although IDO production by DCs could explain the

suppression observed in this study, IDO is an intracellular protein and no secreted form has been described. Thus the effects seen here may have identified a down stream effecter of IDO that is soluble and can be released into the micro environment. This could be due to pathways activated by IDO or it could be the direct action of tryptophan metabolites such as kynureine which has been shown to have effects on T cell viability (Grohmann et al., 2003). Tan et al have speculated that the production of IDO causing suppression of T cell proliferation could be the immune systems way of balancing and restricting the response to adenovirus (Tan et al., 2005). However Tuettenberg et al have shown that it is not merely the infection process that causes the suppression. With the use of "gutless" vectors and inhibitors of protein synthesis it has been shown that a viral product could be responsible and could be another mechanism by which adenovirus can subvert the immune system (Tuettenberg et al., 2004). This may be a more general phenomenon since evidence is emerging that show Herpes Simplex virus infection of DCs having a similar inhibitory effect on T cells in an MLR (Pollara et al., 2003). Despite extensive work in the area and the knowledge that IDO is produced by stimulated DCs and can inhibit T cell proliferation (Hwu et al., 2000) it is not yet clear whether IDO produced by DCs can stimulate the production of Treg in the responding T cell population. It is known that although IDO is constitutively expressed in DCs, T reg expressing the CD80/86 ligand CTLA4 are required to stimulate the DCs to express functionally active IDO (Munn et al., 2004). This may be a mechanism by which Treg function. However it is unclear whether this is part of a positive feed back loop that reinforces the inhibition of the T cell response by producing T reg. Interestingly IL-10 has been shown to enhance the production of IDO overcoming the down regulatory effect pro inflammatory cytokines such as

IFNγ have on IDO production by DCs (Munn et al., 2002). While IDO production by DCs may be involved in the suppression in this system, it is an intracellular protein and no secreted form has been described to date. Further work is in progress to investigate the role of this pathway in our system.

In conclusion, the results presented in this chapter show that in this system the infection of iDC with these adenovirus constructs leads to a population of DCs that are powerfully inhibitory to T cells, and that this is a dominant effect, mediated by soluble factors. The effect is independent of the cDNA inserted (either MV-NP or β-galactosidase genes). The results show that one of the molecules in mediating this effect may be IL-10, at least in some individuals, although they also suggest that the source of this IL-10 is not the virally infected DCs themselves, but perhaps a population of regulatory T cells which have been generated *de novo in vitro*. Work is now in progress to identify both this 'secondary regulator' population as well as the soluble factors which mediate this powerful inhibition. In conclusion caution should perhaps be used in consideration of vectors for the delivery of gene therapy since it is increasingly clear that small differences in constructs may lead to large differences in effects on the immune system.

Chapter 7 Meningitis B vaccine study

7.1 Introduction

7.1.1 *N. Meningitidis* Background

Neisseria Meningitidis is an intra cellular gram negative diplococcus capsulated bacterium (figure 7.1).

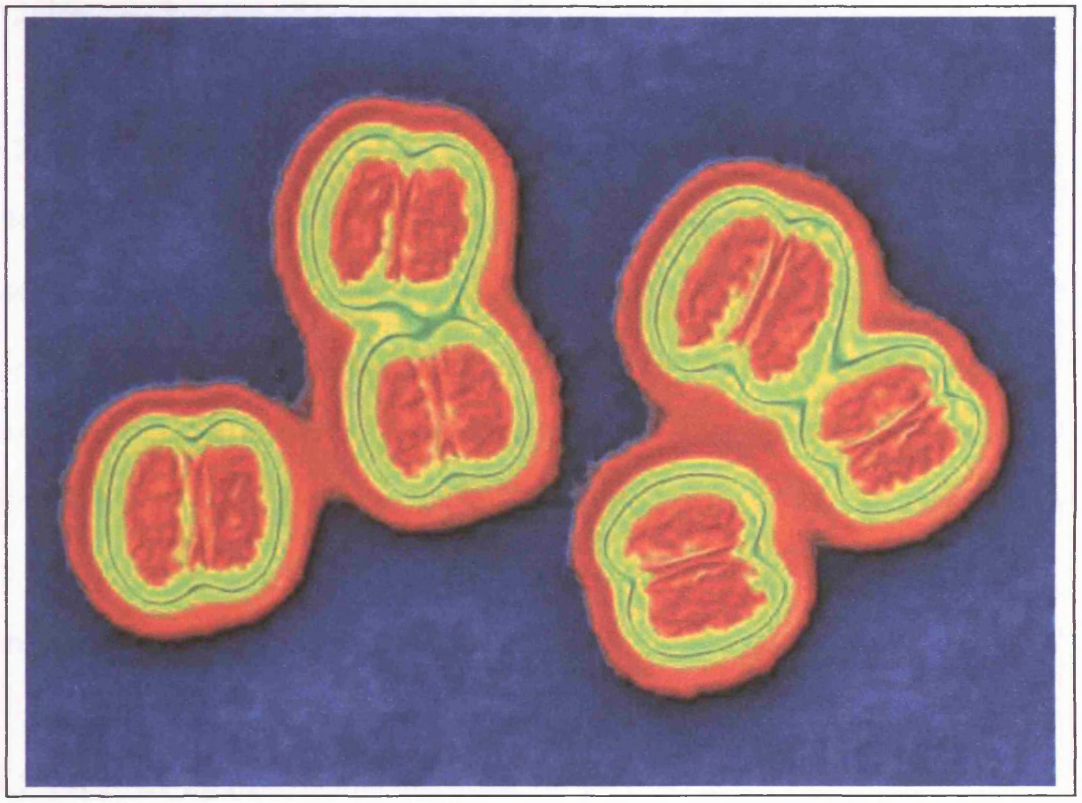


Figure 7.1 N. Meningitidis B coloured electron micrograph picture

It is usually a commensal organism of the nasopharynx (Tzeng and Stephens, 2000). However *N. Meningitidis* is also a major cause of bacterial meningitis and sepsis posing a serious threat to global health and as such contributes to a global annual death rate of 171,000 (WHO, 2002). Despite advanced treatment in industrialised countries case fatality rates are still 5-10% being even higher in the developing world, also leaving 10-12% of survivors with permanent symptoms such as epilepsy and mental retardation (Jodar et al., 2002). There are still epidemics

that are caused by specific serogroups that circulate in regions of the developed world. There is a current epidemic that is affecting New Zealand. Rates of case numbers have risen from 1.5 per 100,000 of population in 1990 to 17.4 in 2001. This epidemic is driven by one strain suggesting that it may be controlled by a strain specific OMV vaccine, a strategy which is currently being implemented by the public health association in New Zealand (Dyet et al., 2005).

7.1.2 Serogroups of meningitis

N. Meningitidis isolates can be divided into 12 groups based on antigenically distinct polysaccharide capsules. Five of these are the pathogenic serogroups A, B, C, Y and W135 (Branham, 1953). In Europe 50-90% of cases are caused by group B and the remainder by group C, where as A predominates in the so-called "meningitis belt" in sub Saharan Africa. The incidence of group B disease is highest in children under one year of age. In contrast C often causes outbreaks in teenagers, army recruits and young adults (Jodar et al., 2002).

7.1.3 Vaccines against N meningitides

In recent times significant efforts have been made to produce effective vaccines against *N. Meningitidis*. Polysaccharide capsules of most serogroups are important determinants of virulence and their purified capsules form the basis of successful vaccines against serogroups A, C, W and Y therefore are important vaccine candidates. They form the basis of polysaccharide vaccines against *N. Meningitidis* strains and other potentially invasive bacteria.

The first successful capsular polysaccharide vaccine against strains A and C was developed over 30 years ago in the USA in response to epidemics among new military recruits. Although a temporary solution to the spread of the disease, these vaccines did not lead to immunological memory and were not effective in young children (Jodar et al., 2002).

Much more recently following the success of the *Haemophilus influenzae* type b (Hib) and pneumococcal conjugate vaccines, immunogenicity of the meningitis capsular vaccines was improved via conjugation of the polysaccharides to a protein carrier, thus inducing a T cell dependent antibody response. This vaccine is also immunogenic in infants and induces long term protection, with the added advantage of reducing carriage and therefore transmission (Goldblatt, 1998) thus increasing herd immunity. The meningitis C conjugate vaccine has been introduced into the UK vaccination schedule and was used in a catch up campaign for everyone in the UK under the age of 18, starting in 1999.

Multivalent conjugate vaccines are now in licensed for A, C, Y and W135 serogroups. However, an effective vaccine against group B is proving to be more of a challenge because the serogroup B capsule has homology with the human neural cell adhesion molecule (NCAM) and therefore is not immunogenic in humans.

7.1.4 Correlates of protection

Traditionally protective immunity against bacterial pathogens has been measured by serum bactericidal activity (SBA) titres, which is considered to be the best

correlate of protection. Serum bactericidal activity is measured either using human or baby rabbit complement. To perform the SBA assay, serial dilutions of human sera are incubated with complement and the target strains of the bacterium. The specific antibodies bind to the meningococcus and the complement component C1q binds to the Fc portion of the Ig. This then activates the classical pathway of complement (as described in chapter 1) which results in the death of the target cell. The number of bacterium present before incubation with serum and complement is compared with the number present after and SBA titres expressed as the serum dilution at which 50% killing of the bacterium occurred (Borrow et al., 2005).

The original gold standard of protection was established by Goldschneider et al in 1969 (Goldschneider et al., 1969b). They bled army recruits at the beginning of their training and determined the SBA titre for serogroup C with human complement. The recruits were followed and monitored for the development of meningococcal disease. Out of the 54 prospective meningococcal cases only 3 had SBA titres of ≥4 compared with 444 of 540 individuals who did not develop disease (5.5% compared with 82.2%). It was also found that as the percentage of children with an SBA titre of ≥4 declined from birth due to the waning of maternal antibodies the incidence of MenC disease increased peaking at 1-2years (Goldschneider et al., 1969a). Thus an SBA titre using human complement of ≥4 was considered to be protective. Later it was recommended by the World Health Organisation (WHO) that in order to further standardise this assay between laboratories baby rabbit complement should be used in a SBA assay for evaluation of meningococcal polysaccharide vaccines. As baby rabbit complement rendered meningococcus more susceptible to lysis than human complement, the assay had

to be re-evaluated this resulted in a SBA titre of ≥8 being considered as protective when using baby rabbit complement (Borrow et al., 2005).

These standards applied well to assessing protection against Meningococcus C infection, however it appeared meningococcus B was harder to evaluate. It appeared that there was a wider variation of SBA titres between laboratories and it may be more appropriate to use a four fold (2 dilutions) rise in SBA titre as an indication that an individual has responded to a meningococcus B vaccine (Borrow et al., 2005;Vermont and van den, 2002). This was validated by Martin et al (Martin et al., 2005) who investigated the antibody response associated with a meningococcus B vaccine trial. They used human complement for this assay that was batch tested to meet certain parameters such as less than 15% kill at 25% concentration. The findings of this investigation were that there was wide variability in the assays at the lowest dilution factors and therefore the lower limit of SBA titre measurable was ≥2. This meant that protection was defined as ≥8 (a 4 fold increase in titre) for individuals with a pre vaccination titre of ≥2 or ≥16 if the pre vaccination level was ≥4 etc. However other correlates of protection should be investigated in the absence of SBA increase as otherwise response to a vaccine could be under estimated in the population (Vermont and van den, 2002).

7.1.5 Interaction with the immune system

N. Meningitidis is constantly circulating within the population, carried in the nasopharynx of colonised individuals, however disease, caused by the organism crossing the mucosa and entering the bloodstream, is relatively rare this suggests that there is a substantial amount of natural mucosal immunity to the organism.

The factors involved in the pathogenesis of this bacterium in certain individuals are at this time still unclear.

Protection is strongly correlated with antibody production, however in order for class switching and affinity maturation to occur, T helper cells must be involved in the immune response. Recent work by the Heyderman group (Davenport et al., 2003) has investigated meningococcus specific T cells in tonsils of human patients. They have found that there are specific T cells in the mucosa which were more abundant, giving stronger proliferative responses to serogroup B derived OMV, the older the individual. They also found that these T cell responses correlated well with anti meningococcus IgG levels but not with bactericidal activity (section 7.1.4). Interestingly more recent unpublished data from this group would suggest that although specific T cells are present in the mucosa, they are hard to detect in the peripheral blood. In contrast, a different group has shown a there is a correlation between SBA titres and T cell proliferation however this was to the PorA protein but not whole OMV after vaccination with an OMV based vaccine (Oftung et al., 1999)

There is also an emerging body of work on the effect of meningococcus outer membrane proteins on the phenotype and function of human dendritic cells. Much work has been done on the effect of LPS on DCs (Dixon et al., 2001;Sprong et al., 2001) which has a wide range of immunomodulatory effects, and can contribute to adverse reactions to vaccines (Geier and Geier, 2002) however it has been shown that outer membrane proteins can also have an effect. Al-Bader et al (Al Bader et al., 2003;Al Bader et al., 2004) firstly showed that exposure to OMV with or without

LPS differentially up-regulated expression of TLR4 and TLR2 on DCs and went on to show that the PorA protein was responsible for this. Singleton et al showed that PorA acted via a TLR-2, MyD88 dependent mechanism (Singleton et al., 2005). The fact that single proteins from the outer membrane of *N. Meningitidis* can activate the immune system is an important observation. As current vaccine development and immunogenicity studies use OMVs derived from the organism which contain all the outer membrane proteins from the surface of the bacterium. This could be a positive effect of vaccinating with OMV, whereby the immune system is activated in an appropriate way by activation through ligation of TLR molecules.

7.1.6 Vaccine strategies for serogroup B

The polysaccharide capsule of *N. Meningitidis* serogroup B is a homopolymer of sialic acid α2-8 N-acetylneuraminic acid. This molecule is structurally and antigenically identical to polysialosyl glycopeptides of the human neural cell adhesion molecule (NCAM), which is expressed in human neural and extra neural tissues (Finne et al., 1983).

This fact makes vaccine development using the group B capsule difficult for two reasons; firstly, the capsule is poorly immunogenic as reactive T and B cells to this molecule would have been deleted during development and the molecule tolerated. Secondly while trying to increase the response to this molecule, there is a risk of raising an immune response against a self-protein which could result in some form of autoimmunity. Thus, alternative vaccine targets for meningitis B are being investigated.

Animal models have been used to investigate the effect of potential vaccine targets on the immune response to *N. meningitides*. As the only natural host for the organism is humans, it is difficult to develop a good animal model. The two main models that are currently used are intraperitoneal infection of adult mice or infant rats. However in order for the animals to develop lethal bacteraemic infection the injection must also include an exogenous iron source such as human transferrin. These models can be used to investigate either direct immunization of the animal using different vaccine candidates and protocols or passive protection provided by human serum from vaccinated or naturally immune individuals. More recent models have used humanized mice that are transgenic for human CD46. These mice do not require an exogenous iron source to be infected and the bacterium can cross the blood brain barrier in these mice but not in wild type mice. This new model would appear to be better that the previous two however as there is only 15% mortality post infection it may be hard to evaluate any vaccine candidates and so perhaps other innate immune mechanisms could be knocked out in these mice to make them more susceptible to meningococcus challenge (Gorringe et al., 2005).

Models such as these have been used to investigate such parameters as verification of OMV vaccination in mice (Quakyi et al., 1999) and to show that 3 doses were required for an increase in IgG avidity while 4 doses induced a larger range of ab specificities. Although the group of mice given the 4^{th} dose when boosted 7 months after the primary dose series, had a reduction in SBA titre but not IgG recall responses, compared to mice given only 2 or 3 does in the primary

series. It was thought that this could be due to the generation of blocking IgG that could block bactericidal killing, although this has not been observed in human studies (Gioia et al., 2005). Studies in mice have shown that intra nasal immunization can induce the same levels of spleen cell proliferation and antibody levels as subcutaneous injection with no evidence of tolerance to these antigens at the mucosal surface, this being very encouraging for future vaccine strategies (Bakke et al., 2001). Animal models have also been used to identify new antigenic targets by vaccinating with individual proteins two of which, a lactate permease (LctP) and an iron acquisition protein (ExbB) elicited protective immunity in mice (Sun et al., 2005). Two separate groups have also shown that the NadA protein which is present in the outer membrane of *N. meningitidis B*, both elicits bactericidal antibodies and is protective in an infant rat model (Comanducci et al., 2002). It also induced SBA and specific T cells in mice following intra nasal vaccination however protective immunity from re-challenge was not proven (Bowe et al., 2004).

Immune responses to specific proteins of meningococcus have also been investigated in humans. Jose et al (Jose et al., 2000) identified predicted peptides from the IgA protease from pathogenic meningococci. 16 out of 20 donors reacted to one of the peptides and T cell lines were grown out that were CD3+CD4+ and were Th1 biased. T cells responses have also been analysed to the class 5 outer membrane proteins OpaB, Opa5d and Opc. This was done using overlapping peptides to the individual proteins to stimulate PBMC and observe proliferation. It was found that the Opa proteins stimulated more proliferation in a larger number of donors and that many of the responses were to the non hypervariable region of the

protein, although no specific dominant epitopes were identified (Wiertz et al., 1996). In a further attempt to identify meningococcal T cell epitopes, a different strategy was used by Meiring et al (Meiring et al., 2005) where an isotope tagged porin A antigen was added to human DCs then purified and analysed as to the sequence of the potential MHC class II epitopes. This method identified several immunodominant regions of the porin A protein that may be important in future sub unit vaccine production.

One of the other alternative strategies for vaccine development utilises the natural blebbing action of the bacterium. Blebbing results in outer membrane vesicles (OMV), which are particles of bacterial membrane and therefore contain all the potential targets that would be on the surface of the bacterium itself. Importantly these are in their natural conformation. The OMV also contain LPS and are treated with detergents to remove as much of this as possible due to the extreme pro inflammatory effects that LPS can have on the immune system (Martin et al., 2005;Sprong et al., 2001). As carriage of the commensal bacterium *N. Lactamica* has been implicated in cross protection to *N. Meningitidis*, OMV from *N. Lactamica* are being used in vaccination strategies that have proved protective in animals and are moving towards clinical trials in humans (Gorringe et al., 2005).

The major protein targets are the porin proteins PorA and PorB which constitute almost 70% of the proteins in the OMV (Naess et al., 1998). Trials of OMV based meningitis B vaccines have showed both production of bactericidal antibodies and stimulation of T cell memory showed by specific T cell proliferation in response to

both OMV and individual porin proteins post vaccination, both in adults (Naess et al., 1998) and to some extent in children (Tappero et al., 1999).

7.1.7 Study plan

Pollard et al (Pollard et al., 1999) studied children recovering from meningococcal disease and postulated that the inability of vaccines to work in the youngest children was due to the immaturity of cellular immunity. They went on to find a trend towards higher proliferative responses in older children, which correlated with an increase in Th2 cytokine production (higher IL-10/ IFNγ ratio). This resulted in the suggestion that any new meningococcal B vaccine may be able to protect young children if it was formulated to stimulate Th2 pathways as well as Th1. Up until recently this is the extent of the work that has been done to characterise the T cell response to meningitis B infection in children in the context of the nature of the response. The aim of this study was to analyse the nature of the immune response to a meningococcal B vaccine. The work presented here is the result of an immunogenicity study of an OMV based meningococcal B vaccine. Alongside SBA titres and T cell proliferation data, using the ELISpot we hoped to further analyse the Th1/Th2 cytokine balance by measuring IL-10, IL-5 and IFNγ. It was also hoped that a more exact phenotype of the cells that were proliferating and producing cytokine in response to the vaccine could be studied using flow cytometry.

7.2 Specific Materials and methods

7.2.1 Meningitis B vaccine

The vaccine preparation that was used for this study was a *N. meningitidis* serogroup B outer membrane vesicle preparation from a specific strain -H44/76. The outer membrane of *N. meningitidis* constantly releases blebs of outer membrane proteins (OMPs) and LPS in their natural conformation. The vaccine is based on these blebs of bacterial membrane or OMV. Chiron provided the vaccine, the exact composition of which is unknown. 10 volunteers received 3 doses of this vaccine at intervals shown in figure 7.2.

7.2.2 Bleeding schedule

The bleeding and vaccination schedule for the meningitis B study is shown below in figure 7.2. 50ml of blood was taken and PBMC and serum were stored as for the MMR study outlined in chapter 4.

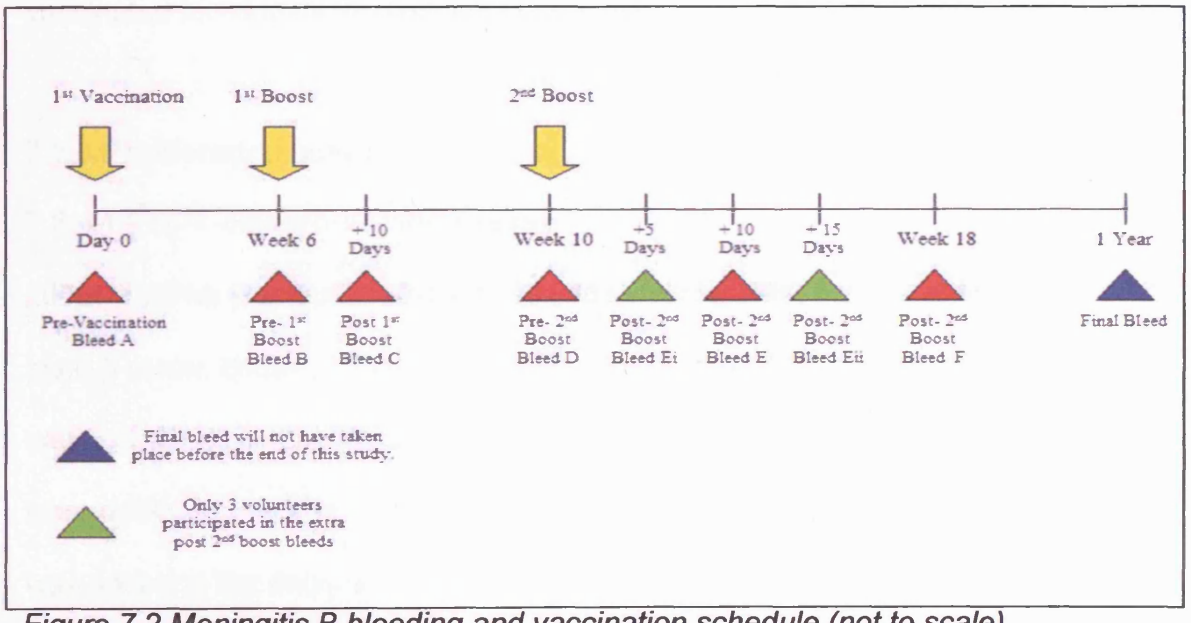


Figure 7.2 Meningitis B bleeding and vaccination schedule (not to scale)

7.2.3 Antigens

The antigen used to detect memory responses in the meningitis B vaccine recipients was the major component of the vaccine (OMV). A related control was also used. The vaccine made use of the natural blebbing process of *N. meningitidis* and consisted of purified outer membrane vesicles (OMV) from one specific strain of *N. Meningitidis* -H44/76. The OMV contain outer membrane proteins and LPS in their natural conformation. The OMV were treated with detergent to remove some but not all of the LPS as when used as a vaccine antigen as outlined in section 7.1, high levels of LPS are not desirable. The two OMV used in the in vitro studies were the relevant OMV derived from the vaccine strain H44/76 and the irrelevant OMV derived from a different *N. meningitidis* strain M0I-240013 The company Chiron and Dr Ray Borrow in Manchester kindly provided the vaccine antigens. They were stored at a concentration of $1mgml^{-1}$ at -70°C. ELISpots were carried out using $1\mu gml^{-1}$ OMV to stimulate PBMC from vaccinated individuals as described in chapter 2.

7.2.4 Proliferation assays

7.2.4.1 [^{3}H]-T-cell proliferation assay

200μl of RPMI was put in all the outer edge wells of a flat-bottomed tissue culture plate (Falcon, Becton Dickinson, France); this was done to prevent artefacts due to edge effect, which can occur in these assays. PMBC at a concentration of 10^6ml^{-1} were added to the other wells 100μl per well. Following this, the antigenic stimulus was added to the designated test wells in quadruplicate. Outer membrane vesicles

(OMV) were used at the same concentration as in the ELISpot. The plate was placed in a humidity box and incubated for 5 days at 37°C/5%CO_2.

7.2.4.2 Pulsing cells

After the 5-day incubation, cells were pulsed with [*methyl*-^{3}H] thymidine (Amersham Pharmice Biotech, Amersham, UK). Each well was pulsed with 1μCi ^{3}H thymidine. The required number of Ci were removed from the stock vial (1μCiμl^{-1}) and diluted 1/10 in PBS and 10μl of this was transferred to each test well. The plate was then put back in the humidity box and incubated at 37°C/5%CO_2 for a further 16 hours. After this incubation the cells were either frozen at -70°C or harvested directly if frozen the plate was removed from the freezer one hour prior to harvesting.

7.2.4.3 Harvesting and data analysis

Cells were harvested using standard protocols on a Harvester 96® (TOMTEC, Wallac, Finland). Radioactivity was measured on a 1450 MicroBeta Trilux counter (Perkin Elmer™, Life Sciences), in counts per minute (cpm) using standard protocols. Proliferation values were expressed in cpm and were calculated as a mean of the quadruplicate unless one obvious outlier was observed.

7.2.5 Limulus Amebocyte Lysate (LAL) assay

The LAL assay was used to semi quantify the endotoxin (lipopolysaccharide, LPS) content within the OMV. This was to rule out any differences in response between the test and control OMV as being due to differences in LPS content

and also to make sure it was not the LPS in the preparations that was causing an *in vitro* response. The assay was carried out using the ETOXATE® kit (Sigma Chemicals Company) following the manufacturers instructions. The assay was carried out in endotoxin free glass culture tubes(Pyrex®, VWR International Ltd, UK). This was prepared using E-TOXA-CLEAN®(Sigma) following the manufacturers instructions. Instead of the standards provided in the kit which would give endotoxin levels in EU, commercially obtained LPS (Sigma) at known concentrations were used. A range of LPS dilutions and OMV concentrations were used, OMV from $1mgml^{-1}$ to $10ngml^{-1}$and LPS from 1μg to 1pg. All dilutions were made in the endotoxin free water provided with the kit and all controls were prepared as directed by the instructions. A positive test was the formation of a hard gel and from the dilution series prepared, a semi quantitative endotoxin level could be calculated.

7.3 Results

This study was done in collaboration with the Health Protection Agency (HPA) and some of the laboratory work was done jointly with Miss Ihjaaz Quireshi, a visiting Masters student from the London School of Hygiene and Tropical Medicine. Ihjaaz was taught to do proliferation assays and then generated the bulk of the H-thymidine proliferation assay data. The ELISpot had to be optimised before the study began for the OMV antigen, as it had been originally optimised for peptide antigens.

7.3.1 OMV and irrelevant OMV titrations

It was known that some individuals in the pre vaccination bleed would already respond to some antigens in the OMV, including LPS. Thus titrations of both the relevant and irrelevant OMV were carried out in unvaccinated healthy donors to ensure the correct concentration was being used for optimal response. The results of the titration to the relevant OMV in 3 healthy donors are shown in figure 7.3. Throughout the subsequent study OMV was used at a protein concentration of $1\mu gml^{-1}$.

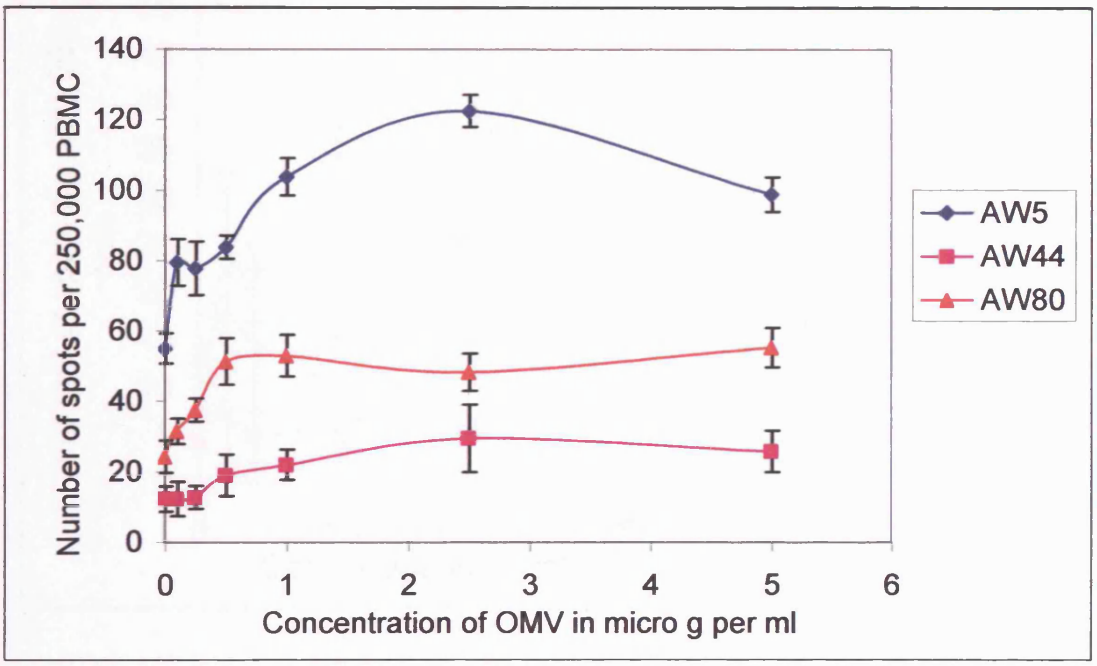


Figure 7.3: Titration of relevant OMV in 3 healthy donors in the ELISpot with 250,000 PBMC per well, showing an optimal response at $1\mu gml^{-1}$. Data points represent mean and standard deviation from triplicate wells.

7.3.2 IL-5 ELISpot optimization

As it was T cell help that was being investigated in this trial and not only Th1 but also Th2 responses would be expected, an IL-5 ELISpot was developed. As the responses were so low from unvaccinated donors, a titration of responses by atopic donors to dust mite antigen was carried out to ensure the IL-5 ELISpot was working. One donor made a clear positive dose response to the antigen (AW81) shown in figure 7.4.

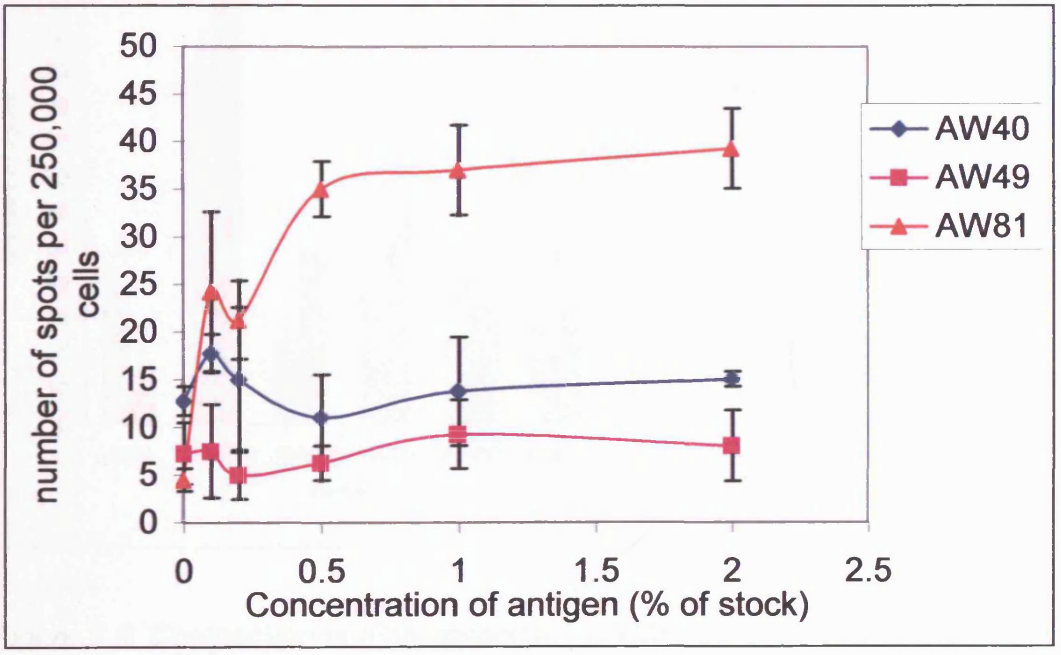


Figure 7.4: Results of an IL-5 ELISpot with 250,000 PBMC per well from 3 atopic donors stimulated with dust mite antigen at increasing concentrations. Mean and standard deviation are shown from triplicate wells. This was used as a positive control on all IL-5 plates in future experiments. Values are mean of 3 wells and error bars represent 1SD.

7.3.3 Responses to OMV in fresh and frozen PBMC

As the samples were to be stored for use at a later time, to enable the responses at different time points post vaccination to be compared simultaneously, the response of fresh and frozen cells to OMV was compared in figure 7.5 to ensure there was no difference. A student's paired T test was done and no significant difference was found between the fresh and frozen samples p>>0.1.

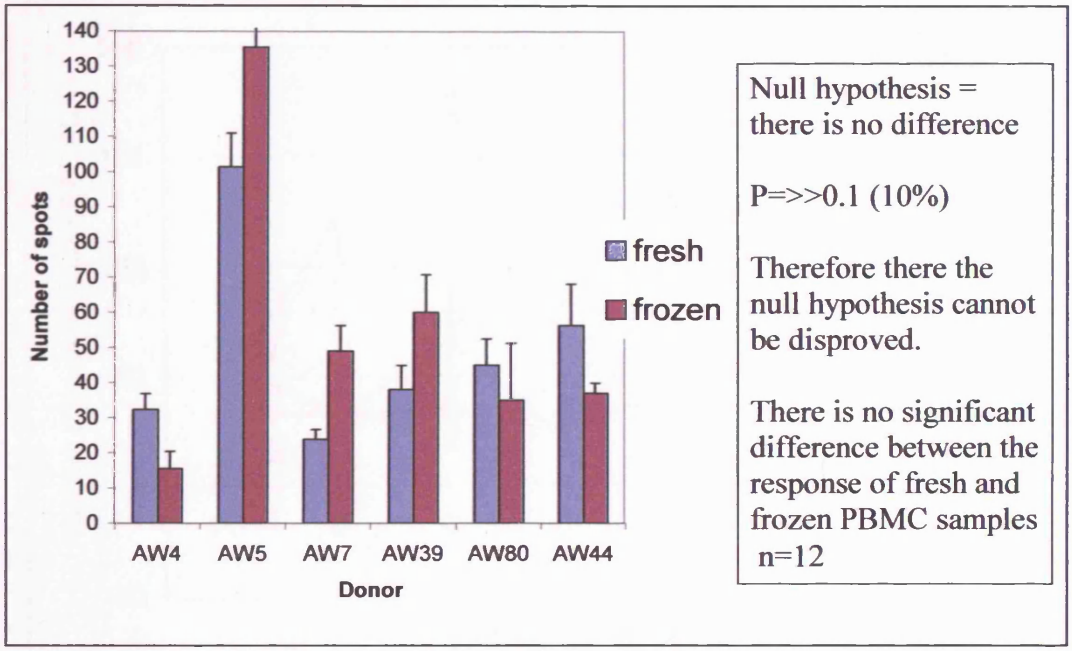


Figure 7.5: Comparison of IFNγ responses to OMV by fresh and frozen PBMC as measured in the standard ELISpot in 6 adult volunteers with 250,000 cells per well. Mean and standard deviation are shown from triplicate wells.

7.3.4 Study results

All of the samples from the different time points from each volunteer were compared in the same assay after freeze thawing, to avoid any inter-assay variation in both the ELISpot and proliferation assays.

7.3.4.1 ELISpot

The results from both IL-5 and IFNγ ELISpot assays showed no significant alteration in response between pre and post vaccination although there seemed to be a transient boost in T cell production of IFNγ at time point C- 10 days post vaccination, in some individuals as shown in figure 7.6 (IFNγ ELISpot) and 7.7 (IL-5 ELISpot).

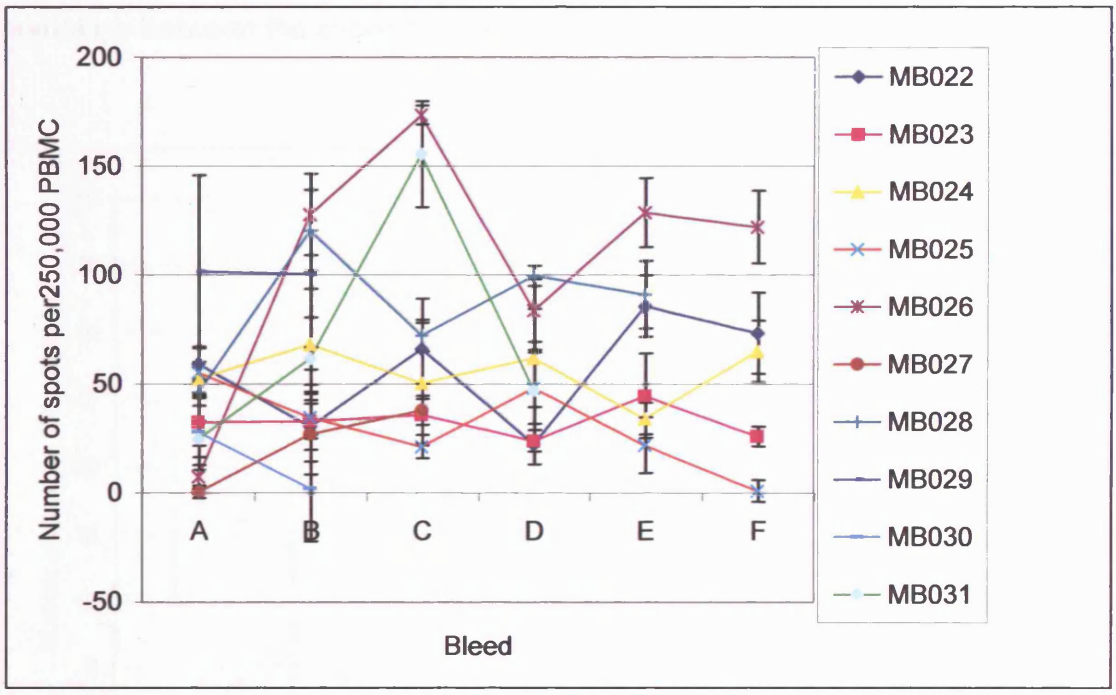


Figure 7.6 Summary of responses of vaccinated individuals to the relevant OMV preparation by production of IFNγ over the course of the vaccination schedule. Data points show the mean and standard deviation from quadruplicate wells after the response to medium alone was subtracted.

In these experiments OMV from the vaccine strain and an irrelevant strain of meningococcus were tested. Figure 7.6 shows the IFNγ response to the vaccine strain OMV however the response to the irrelevant strain of OMV appeared to be higher than the response to the vaccine strain in most vaccinated individuals (data

not shown). Therefore OMV preparations were tested for endotoxin levels and were found to be smaller in the irrelevant OMV than in the relevant OMV, so the higher response of vaccinees to the irrelevant OMV could not be attributed to endotoxin levels. This result may reflect a higher number of cross reactive epitopes between commensal species such as *N. lactamica* or more common non pathogenic circulating strains of *N. meningitides* and the M0I-240013 strain of bacterium used, however a more detailed study than this, looking at protein homology between the species would need to be carried out to confirm this.

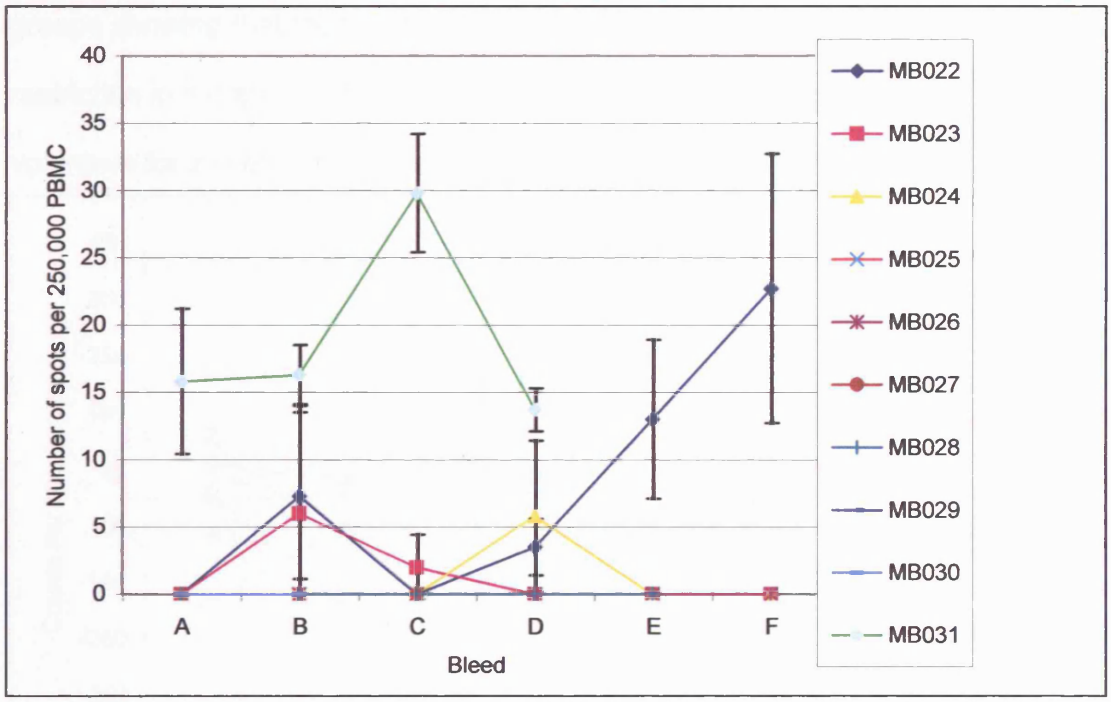


Figure 7.7 Summary of responses of vaccinated individuals to the relevant OMV preparation by production of IL-5 over the course of the vaccination schedule. Data points show the mean and standard deviation from quadruplicate wells after the response to medium alone was subtracted.

7.3.4.2 Proliferation assay

No significant change in proliferation to the OMV could be detected over the course of the vaccination schedule in any of the donors as shown in figure 7.8. Proliferation assays were carried out as outlined in section 7.2 with samples assayed in triplicate. Very low levels of proliferation were seen with often higher proliferation in control wells than in antigen stimulated wells, this is why there are negative values for cpm in figure 7.8. Prior to study samples being assessed, healthy volunteers were used to optimise the proliferation assay methodology. Comparable responses were seen to PHA and Tetanus toxoid antigens as other groups showing that the proliferation assay was working (data not shown). Due to restriction in sample sizes- approximately 60million cells per time point per volunteer for all cell based analysis, all these experiments were performed once.

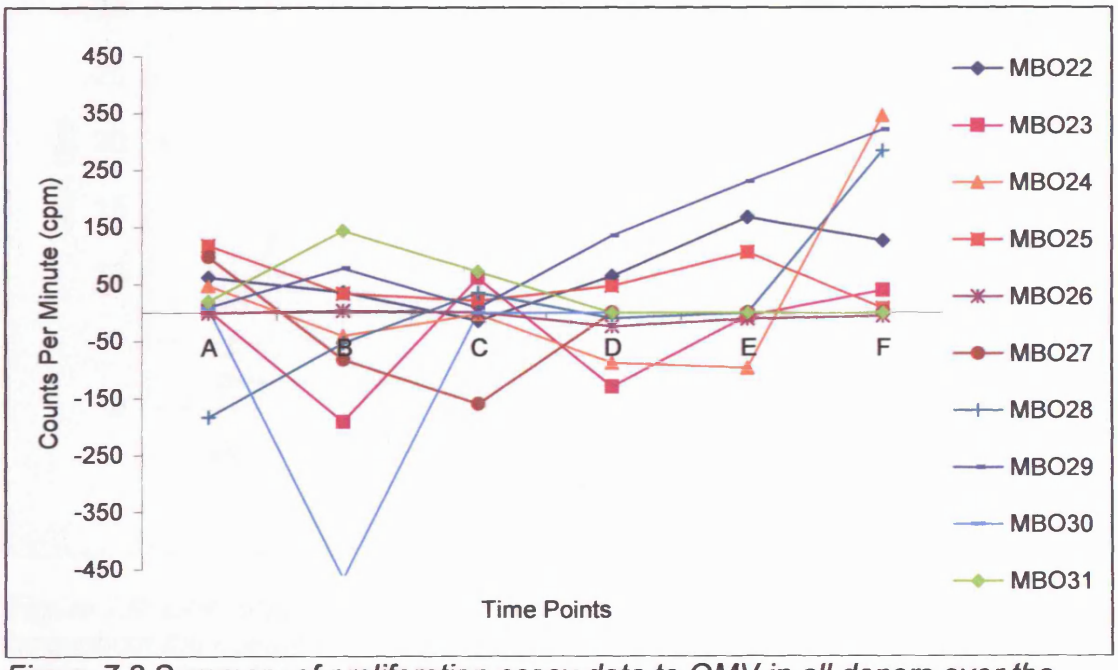


Figure 7.8 Summary of proliferation assay data to OMV in all donors over the vaccination schedule. The values of cpm were calculated by subtracting background proliferation from proliferation to the antigen. All values are mean of 3 wells SD are not shown for ease of viewing. None of the changes were statistically significant when a student's t test was used to compare pre and post vaccination proliferation.

7.3.4.3 SBA titres from study participants

Serum samples were also taken throughout this study and analysed for specific SBA activity by collaborator Dr Ray Borrow in Manchester. The result of this analysis was disappointing and shows that few of the vaccinated individuals responded to the vaccination programme. As outlined in section 7.1.4, response to vaccination against serogroup B of meningococcus may involve more than only SBA activity, however this study failed to show a cellular response to vaccination. A significant response to vaccination when measuring SBA titres is considered to be a four fold (2 dilution factor) rise in activity pre and post vaccination.

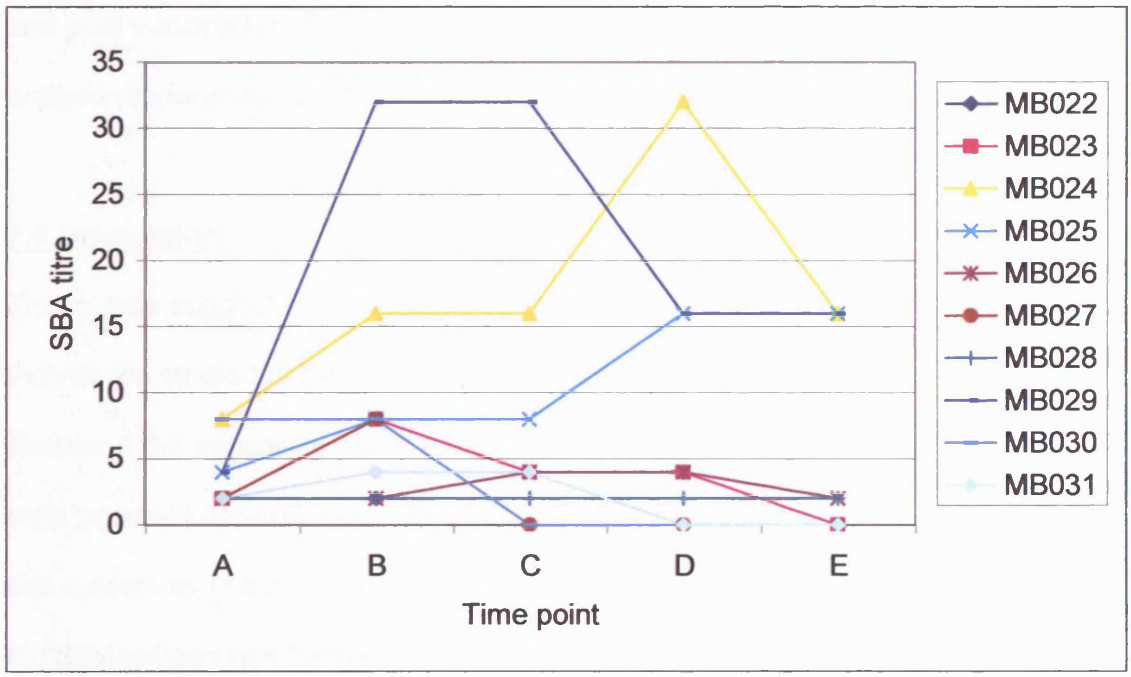


Figure 7.9: Line graph to show SBA titre to the vaccine strain of N. meningitidis, throughout the course of the vaccine schedule. Serum from vaccinated individuals was incubated with complement and the bacterium at increasing dilutions. SBA titre represents the serum dilution at which 50% of the bacteria are killed. A positive response to the vaccine is considered to be a 4 fold rise in SBA equivalent to an increase in SBA of 2 dilutions. Only MB024 and MB029 are considered as responder to this vaccine by SBA titre.

Figure 7.9 shows the SBA titres for the vaccinees over the course of the vaccination schedule. These data were provided by Ray Borrow and colleagues in Manchester. According to the parameters – an increase in SBA of 2 dilutions considered significant, only 2 individuals responded to this vaccine, MB024 and MB029. When looking at the proliferation assay data both these individuals have positive proliferation of T cells in response to OMV at the later points in the vaccination schedule which is encouraging, however an increase post vaccination in cytokine production was not detected in these individuals. SBA titres to several other strains of meningococci were measured and showed similar results to figure 7.9. There were very few individuals whose SBA titre increased 4 fold between pre and post vaccination. This shows there was also minimal boosting of shared antigen responses with this vaccine (data not shown).

7.4 Discussion

These data suggest that there has been little or no response in the vaccinated individuals studied to the OMV vaccine against *N. meningitidis B*. As discussed in chapter 4 the sensitivity of the assay is potentially 1/250,000 cells (the number of cells per well) depending on the background (non specific spot formation). Thus in this system as few as 24 specific cells per million would be detectable. Proliferation to OMV antigen has been detected in vaccinated individuals, although the precursor frequency of responding cells is hard to extrapolate from a thymidine incorporation proliferation assay. In addition to this, not all of the cells that proliferate to OMV will produce cytokine but a response of some level with the production of Th1 or Th2 associated cytokines would be expected. Proliferation was also measured in this study in the same way as in other studies that had

showed positive results, however a boost in proliferation to OMV was not observed in these donors and SBA titres were not boosted in most individuals. It is therefore likely that there was not a response to be detected in the individuals vaccinated in this study.

Significant responses to OMV vaccination have been recorded by measuring SBA titres in vaccine trials both large and small. Tappero et al reported the results of a vaccine trial in Chile. However the percentage of vaccinees that responded to the vaccine strain of *N. meningitidis* was very small –only 35% and children under 4 years of age did not respond at all (Tappero et al., 1999). A similar disappointing result was recorded by Katial et al where less that 50% of vaccinees responded by an increase in SBA levels (Katial et al., 2002). However two more recent studies have shown more encouraging results one group showing that SBA titres, opsonophagocytic activity, class switching and affinity maturation of antibodies are increased post vaccination however only after intra muscular vaccination but not intra nasal vaccination (Aase et al., 2003). Results of the OMV vaccine trial in New Zealand show that the vaccine is well tolerated and 96% of adults and 74% of children responded with a four fold increase in SBA (Oster et al., 2005). There are only three studies by 2 groups that have so far investigated the T cell response to OMV vaccines against *N. meningitidis* B. One of these studies has shown proliferative responses in three vaccinees to an OMV vaccine containing 6 different PorA proteins from 6 different target strains along with boosting of specific antibody responses (van der Voort et al., 1997). Studies conducted in Norway have shown significant rises in levels of PBMC proliferation post vaccination that can be boosted with further doses of vaccine, and that these responses are predominantly

to the PorA protein of *N. Meningitidis B*. They show this for both intra muscular and intra nasal routes of vaccine delivery (Naess et al., 1998;Oftung et al., 1999). However still not all of the vaccinees respond and responses to whole OMV in some cases are very low compared to PorA responses with this especially true after intra nasal vaccination. The latter two studies used the same vaccine that was used in the study outlined above.

The reason for the lack of detectable response in this study is at present unclear. It could be that the sample size of vaccinees was not large enough to account for so many non responders, or as this vaccine has been used successfully in the past, it could be that there was a problem with the vaccine batch or inappropriate storage which could not have been foreseen at the start of this study, and caused the failure of the vaccine. The data generated in this study can only suggest that this vaccine produced no induction of T cell immunity in the 10 individuals studied and did not promote a protective boost in SBA levels in 8 out of 10 vaccinees.

Further work on the remaining samples from this study could be undertaken, such as pre incubation of autologous DCs with OMV prior to performing proliferation assays and ELISpot assays to allow processing and more efficient presentation of the OMV antigen to the T cells which may boost observed responses. CFSE assays could also be carried out in place of the proliferation assays using H thymidine incorporation this would allow phenotypic identification of proliferating cells, as currently PBMC have been used in all the studies mentioned so it is not actually proven that it is T cells and not B cells or monocytes proliferating to the OMV antigen. However as the vaccine does not appear to have boosted even SBA

titres in most individuals studied there may not be any T cell responses to observe, even with renewed effort and modified methods aimed at boosting observed responses. Yet the original questions still stand- there must be *N. meningitidis B* specific T cells present in immune individuals as they are required for class switching and affinity maturation of specific antibodies therefore, what is there phenotype? Which cytokines do they produce? and are they boosted by vaccination? In order to answer these questions further vaccine immunogenicity studies are under way with different OMV based vaccines which it is hoped will show boosts in SBA titres and therefore allow a full investigation in to the nature of the T cell response against *N. meningitidis B.*

Chapter 8 Conclusions and future work

8.1 Measles epitopes

The aim of the work described in chapters 3, 4 and 5 was to discover peptides derived from the measles virus (MV) that are recognised by human T cells from immune donors, in the context of HLA-A2*0201. Firstly, peptides that had been identified by other groups were investigated, to test their ability to stimulate T cells to produce IFNγ, as assessed by ELISpot. Before this could be done, the ELISpot assay was set up and optimised for the identification of low frequency cells in this system. Although several strategies were investigated to try to increase the detection of antigen specific responses to the predicted peptides, no amplification of responses was seen over background in MV-immune individuals. This may have been due to very low precursor frequencies of responder cells and in light of the negative results a better approach may have been to measure T cell proliferation to whole MV, individual proteins and peptides to be investigated. This would have had two benefits, firstly it may have amplified the signal to ensure that there was a response to be measured in the donors used in the study, and secondly may have identified the individuals who had the highest responses, prior to quantification of this response using the ELISPOT. Due to the negative nature of the first, more specific approach to epitope identification, a broader approach was developed, where pools of overlapping 15mer peptides derived from MV proteins F (fusion), NP (nucleoprotein), and H (haemagglutinin) were used to stimulate PBMC from immune donors. This section of the work was presented in chapter 3.

The first strategy did not identify any definite epitopes. It is known from previous studies of viral immunity that precursor frequency of memory CD8 T cells may be in the range of 40 per million in the case of influenza epitopes (Rehermann et al., 1996) to 2,500 responders to one peptide detected per million PBMC in EBV infected patients (Tan et al., 1999). The limit of detection of the ELISpot over background is typically over 1 in 40,000. Therefore, in theory if memory T cells to MV exist at this frequency or above, they would be detectable in this assay.It is possible that memory cells which are specific for the chosen peptides are present but at a frequency below the detection limit of the assay. Alternatively these may not be immunodominant epitopes for the HLA-A2 donors.

Due to the inability to identify MV epitopes, a new strategy was developed which is discussed in chapter 4. This involved the vaccination of two adult volunteers with the MMR with the aim of transiently boosting responses *in vivo*, which could have enabled the identification of epitopes due to increased precursor frequency of responding cells. PBMC from different time points post vaccination were stimulated with MV peptide pools in the ELISpot. This strategy enabled identification of some pontentially interesting sequences. However the number of responder cells was so low that a confident identification of HLA-A2 specific epitopes could not be made. The results are difficult to interpret given that vaccination was in only two individuals. This was the weakness of this section of the work, for confident identification of HLA-A2 epitopes it would have been necessary to recruit more individuals for vaccination with the MMR. This was not done in the first instance, as once interesting sequences had been identified in adults the intention was to study them in a larger number of recently vaccinated children. The epitopes of interest

would need to be identified first however as the size of the blood samples from children would be too small to scan for new epitopes. Thus if this work was to be repeated more adult volunteers should be vaccinated in the initial part of the study and T cell proliferation to whole MV measured in each prior to stimulation with peptide pools.

In a study of frequency of memory T cells proliferating to MV in immune adults, the mean frequencies of memory T cells were 0.35% for CD4+ and 0.24% for CD8+ cells (Nanan et al., 2000). The donors used in this study had had natural measles infection and although the donors used in the study described in this thesis were believed to have had measles in childhood, the specific cells may not have been boosted by vaccination. Furthermore in proliferation assays carried out using PBMC from both pre and post vaccination after stimulation with MV (see section 4.3.2), no clear T cell proliferation was demonstrated. Thus it is possible that these donors had a poor response to the MMR boost given. In a study of responses just after natural MV infection (Jaye et al., 2003), responses were very strong to one epitope from the MV core protein in the weeks after infection however were short lived and failed to persist to memory. This may be a reason for the inability of this study to detect such memory responses in the boosted individuals who had had natural MV infection during childhood. Therefore a method was developed by which DCs from the vaccinated individuals could be made to express individual MV proteins at high levels, by infection with adenoviral vectors containing the cDNA coding for these proteins. This would enable the *in vitro* boosting of T cell responses by the culture of T cells with DCs that had processed and presented individual MV proteins in order to grow short term T cell lines.

Others have used vaccinia viral vectors containing measles proteins for similar studies to infect target cells with MV antigens to observe MV protein specific killing by CTL (Whittle 1998). In retrospect these constructs could have been used for the purpose of infecting DC and expanding autologous T cell lines. The adenoviral constructs however had been used to vaccinate mice and had shown protective responses (Fooks, 1995). Also there was no indication in the literature that adenoviral infected DC would not stimulate T cell proliferation. Therefore the adenoviral vectors were used as protocols were set up in the laboratory for working with adenovirus and not vaccinia at this time. In order for this method to be utilised, the protocols had to be optimised and methods for verification of infection and protein expression were developed. These were successful and protein expression was demonstrated in both monocytic cell lines and human DCs. The effect of infection on DC phenotype was also investigated and in line with the findings of others (Miller et al., 2002;Rea et al., 1999) a trend towards a more mature phenotype was observed. However, no one molecule was consistently up or down-regulated in response to adenovirus infection. Phenotypically the DCs were not changed enough by adenoviral infection to suggest that infection would prevent their ability to stimulate T cells to proliferate, or to act as efficient antigen presenting cells to enable culture of short term T cell lines. The viability of the DCs was also investigated and there was no increased death in adenovirus infected DCs than in uninfected DCs. However the proliferation of T cells to infected DCs was much lower than might have been predicted, and it therefore remained difficult to identify a measles specific response in the T cell lines re-stimulated with peptide pools derived from MV. This was the basis of the data presented in chapter 5.

Despite the difficulty in identification of T cell epitopes derived from the MV virus, this is still a very interesting and important area of work as discussed in section 1.3. As for the future direction of this work, the approach used by the van Els group (van Els et al., 2000) which shows identification of naturally processed epitopes by purification of epitopes for MV infected cell lines via microcapillary high performance liquid chromatography-electrospray ionization-tandem mass spectrometry may be an important method. If this could be adapted for use with primary cells such as DCs either infected with MV or using the same methodology as the adenovirus work presented here, using viral vectors to express individual MV proteins within DCs to allow the identification of naturally processed epitopes. Once epitopes have been identified by this method they can go on to be tested in ex vivo assays to stimulate PBMC taken from both recently vaccinated and MV-infected children. This would also allow for a comparison in responses to these epitopes in the two groups and to gain further information on the difference between vaccine induced and natural immunity.

8.2 Dendritic cells and adenovirus

During the investigation which involved using DCs infected with adenoviral vectors containing MV proteins, outlined in chapter 5, the observation was made that T cells stimulated with DCs that had been infected with adenovirus consistently proliferated less than cells proliferating to uninfected DCs. This phenomenon was therefore further investigated, and this work is the basis of the data presented in chapter 6. Firstly the question of adenovirus making the DCs less efficient APCs was addressed by using PHA to stimulate the proliferation of T cells. This

stimulation does not typically require additional DCs to stimulate proliferation of PBMC, since PHA is a T cell mitogen. This showed that the reduction in the proliferation of the T cells was due to an active suppression by the adenoviral infected DCs, rather than it being the DCs that were defective (see section 6.3.4).

The suppression observed was shown to be due to a soluble factor or factors with the use of transwell and supernatant transfer experiments (section 6.3.7). Blocking experiments showed that in one donor, IL-10 was partly responsible for the suppression observed. However investigation into the production of cytokines by DCs after adenoviral infection, using the supernatants from adenoviral infected DCs compared to uninfected DCs failed to show a significant increase in IL-10 production by DC post adenoviral infection. Analysis of supernatants from adenoviral infected DC/T cell co-cultures did however show an increase in IL-10 with adenoviral infected DCs compared to uninfected DCs. This may imply that adenoviral infected DCs are able to promote the production of Treg -Tr1 cells. Tr1 cells are typically defined by their ability to suppress other T cell responses and their production of IL-10 (Jonuleit and Schmitt, 2003). Several methods are described to generate such Tr1 cells in vitro (Barrat et al., 2002;Kemper et al., 2003;Levings et al., 2005). Whether this system using adenovirus infected DCs may be generating such Tr1 cells, remains to be investigated.

It was also shown that other cell types could mediate the suppression when infected with adenoviral constructs although to a lesser extent which may reflect the cell type's ability to make one or more secreted suppressive factors. The addition of IL-2 did not overcome the suppression, nor did blocking TGFβ. This is

interesting given that both IL-2 consumption and TGFβ have been implicated in the mechanisms of action of Treg (von Boehmer, 2005).

Recent reports have implicated IDO as having a role in adenovirus induced suppression of T cell proliferation (Tan et al., 2005;Terness et al., 2005). Preliminary investigations in this study have not shown that IDO has an effect (section 6.3.11) and as IDO is not known to have a soluble form, the data presented here may have identified a down stream effector molecule of IDO. However this remains to be further investigated in the system described here. In addition to the investigation of the role of IDO, future work on this project would involve the characterisation of the T cells resulting from a proliferation assay with adenovirus infected DCs. Both phenotype and intracellular cytokine production could be analysed using flow cytometry. In order to evaluate the hypothesis that suppression may be due to the generation of Treg, secondary proliferation assays could be carried out using T cells from the primary proliferation assay to co-culture in a secondary proliferation assay. This would allow the investigation of any secondary suppressive effect that the T cells generated by co-culture with adenovirus infected DCs may possess. Another interesting area of work would be the generation of deletion mutants of the adenoviral vector. It has been shown that "gutless" vectors do not have a suppressive capacity (Tuettenberg et al., 2004). Thus the use of deletion mutants would enable the identification of the specific area of the viral genome responsible for the suppression. In the future, this may also enable the identification of the molecule responsible for the suppression and therefore investigation into this molecule as a potential therapeutic agent.

The work presented here is only in a relatively small number of donors. Therefore in order to verify these results and investigate a mechanism of suppression, further donors need to be tested in this system. There are many ways as outlined above in which this work should be expanded on and further verified, which due to time constraints were not addressed in this thesis.

8.3 Meningitis B vaccine development

The work presented in chapter 7 shows the results of an immunogenicity trial of an OMV based meningococcal B vaccine. IFNγ and IL-5 production in response to OMV stimulation was measured by ELISpot and proliferation to OMV was also measured by thymidine incorporation. The results of the trial did not show a boost in T cell responses to vaccine antigen. However SBA titres which have shown to be boosted in other studies were not boosted in majority of individuals vaccinated in this study. Other studies however have observed a T cell response. One of these studies recorded T cell proliferative responses in three vaccinees to an OMV vaccine containing 6 different PorA proteins from 6 different target strains along with boosting of specific antibody responses (van der Voort et al., 1997). Also studies conducted in Norway utilising the same vaccine as used for this study, have shown significant rises in levels of PBMC proliferation post vaccination that can be boosted with further doses of vaccine, and that these responses are predominantly to the PorA protein of *N. Meningitidis B* (Naess et al., 1998;Oftung et al., 1999). However, not all of the vaccinees respond to vaccination. The reason for the lack of detectable response in this study is at present unclear. It could be that the sample size of vaccinees was not large enough to account for so many non responders, therefore if this was to be repeated a larger study population

should be used. Alternatively, as this vaccine has been used successfully in the past it could be that this specific batch of vaccine may not have been effective in the individuals that were vaccinated in this study.

As the question surrounding the phenotype and quantity of meningococcal B specific T cells, remains unanswered, larger future studies could be carried out which may account for the fact that a large proportion of people in the study may not respond to the vaccine. It may also be interesting to use individual MenB proteins to stimulate PBMC in both the ELISpot and proliferation assays, as studies have shown a greater boost to T cell proliferation when re-stimulated with the PorA protein rather than to whole OMV (Naess et al., 1998;Oftung et al., 1999). This may reflect an inability of PBMC to efficiently process and present whole OMV and work is in progress to investigate whether autologous DC pulsed with vaccine derived OMV will allow observation of cytokine production in the individuals that appeared to respond to the vaccine by SBA titres. In addition to this different OMV meningococcal B vaccines are also currently being investigated. PBMC taken from donors in trials using these vaccines may allow identification of the phenotype of responder T cells in both cytokine release and proliferation assays using CFSE so cell surface markers on responder cells can be identified.

In conclusion, these studies have used several different approaches to investigate the human T cell response to a virus (measles) to which protective memory immunity is present, and to a new vaccine product, of a bacterial pathogen. Although the studies failed to identify clear MV epitopes, or the phenotype of cells responding to *N. Meningitidis* B derived OMV, the work did generate a novel and

exciting set of data, and a system in which suppressive effects of DCs may be further investigated in the future.

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Appendix

Table of all "adult well" volunteers used in this study, their date of birth, HLA-type and anti MV antibody titre. Where n/d is used it represents not done.

AW#	DOB	(mIU/ml)	HLA typing
4	07/10/1968	1211	A26,11/B8,57
5	26/06/1960	3414	A2,24/B7,50
7	12/06/1967	9221	A1,2/B57,51
27	16/06/1977	5498	A11,24/B35,51
37	28/06/1971	12634	A2/B07,44/DR0103,15
39	12/03/1969	1176	A2,24/B15,51/DR8,11
49	06/01/1978	5217	A2,26/B8,1501/4,C0302/4,0701/6,
69	17.9.1958	31705	A2/B07,51/C0702,14
73	30.7.1974	1468	A2
75	28/04/1969	11035	A2,68/B1501/4,56/C0102-4,0302/4
76	29/08/1969	7683	A2,32/ B8,1516/17 C0302/4,14
80	27/12/1972	n/d	A11,33/B38,44/C1302,1303/4
81	18/03/1980	17062	A2,29/B27,44/C0202,0501
84	17/06/1971	391	A2,1/B7,35/C0702,04/18
85	09/02/1974	3057	A2,25/B7,18/C0702,1203/6
87	21/11/1979	n/d	n/d
88	04/05/1970	n/d	n/d
89	26/02/1977	5483	A2
90	12/10/1974	n/d	n/d